Bohn, Brent 1166

From:	Sams, Reeder
Sent:	Tuesday, April 01, 2014 10:07 AM
To:	Cowden, John; Lee, Janice; Powers, Christina
Cc:	Cogliano, Vincent
Subject:	Arsenic Materials for Bimonthly
Attachments:	Inorganic Arsenic Materials and Topics for Discussion - revised draft - 03 27 14 VC comments.docx

John et al.,

Vince and I talked about some additional comments that he had for the draft that we sent last Thursday. The comments are included in the attached document. If you have questions we can discuss further.

Best Regards,
Reeder

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Bohn, Brent 1172

From: Joca, Lauren
Sent: Monday, April 28, 2014 11:40 AM
To: Cowden, John
Cc: Powers, Christina
Subject: RE: Materials for ECM/Wound repair MOA

Thanks John! Have a great afternoon!

From: Cowden, John
Sent: Monday, April 28, 2014 11:31 AM
To: Joca, Lauren
Cc: Powers, Christina
Subject: Materials for ECM/Wound repair MOA

Hi Lauren,

Happy Monday! I hope that things are going well for you today.

Here is the draft AOP table for the ECM/wound repair MOA for iAs. I've also sent the template for the write-up section. Christy can probably send you an example from one of the other write-ups.

Let me know if you have any questions. Have a great afternoon!

John

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Bohn, Brent 1173

From:	Cowden, John
Sent:	Monday, April 28, 2014 11:31 AM
To:	Joca, Lauren
Cc:	Powers, Christina
Subject:	Materials for ECM/Wound repair MOA
Attachments:	2014 03 31 MOA Table Vascular Remodeling - draft.docx; 2014 03 05 MOA Text Template.docx

Hi Lauren,

Happy Monday! I hope that things are going well for you today.

Here is the draft AOP table for the ECM/wound repair MOA for iAs. I've also sent the template for the write-up section. Christy can probably send you an example from one of the other write-ups.

Let me know if you have any questions. Have a great afternoon!

John

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Review Article

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New developments in the evolution and application of the WHO/IPCS framework on mode of action/species concordance analysis†

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ABSTRACT: The World Health Organization/International Programme on Chemical Safety mode of action/human relevance framework has been updated to reflect the experience acquired in its application and extend its utility to emerging areas in toxicity testing and non-testing methods. The underlying principles have not changed, but the framework's scope has been extended to enable integration of information at different levels of biological organization and reflect evolving experience in a much broader range of potential applications. Mode of action/species concordance analysis can also inform hypothesis-based data generation and research priorities in support of risk assessment. The modified framework is incorporated within a roadmap, with feedback loops encouraging continuous refinement of fit-for-purpose testing strategies and risk assessment. Important in this construct is consideration of dose–response relationships and species concordance analysis in weight of evidence. The modified Bradford Hill considerations have been updated and additionally articulated to reflect increasing experience in application for cases where the toxicological outcome of chemical exposure is known. The modified framework can be used as originally intended, where the toxicological effects of chemical exposure are known, or in hypothesizing effects resulting from chemical exposure, using information on putative key events in established modes of action from appropriate *in vitro* or *in silico* systems and other lines of evidence. This modified mode of action framework and accompanying roadmap and case examples are expected to contribute to improving transparency in explicitly addressing weight of evidence considerations in mode of action/species concordance analysis based on both conventional data sources and evolving methods.

Keywords: key events; mode of action; adverse outcome pathway; human relevance framework; modified Bradford Hill considerations; weight of evidence approach; species concordance analysis; cellular response; tissue response; molecular target

Introduction

The mode of action/human relevance framework was developed in initiatives of the International Programme on Chemical Safety (IPCS) of the World Health Organization (WHO) (Boobis *et al.*, 2006, 2008; Sonich-Mullin *et al.*, 2001) and the International Life Sciences Institute Risk Sciences Institute (ILSI-RSI) (Meek *et al.*, 2003; Seed *et al.*, 2005). It derives from earlier work on mode of action in animals by the US Environmental Protection Agency (US EPA, 1996, 2005a) and has involved large numbers of scientists internationally.

Previous development of the mode of action/human relevance framework is described in the publications mentioned above and summarized more recently in Meek and Klaunig (2010). The framework has been illustrated by an increasing number of case studies (more than 30 currently) demonstrating the value of mode of action in evaluating human relevance and life stage susceptibility and guiding dose–response assessment. Documented examples are presented in Table 1. The contribution of the framework has been recognized by the Society of Toxicology, and the framework has been adopted by several international and national organizations and agencies to increase transparency in the assessment of weight of evidence and identification of critical data needs (Meek, 2008, 2009; Meek *et al.*, 2008).

The framework continues to evolve as experience increases in its application to consider systematically the weight of evidence

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† This publication contains the collective views of an international group of experts and does not necessarily represent the decisions or the stated policy of the World Health Organization or the authors' affiliated organizations.

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Table 1. Case studies illustrating various modes of action and implications for dose–response assessment

Mode of action	Case study	Reference
Tumors of various organs associated with mutagenic modes of action	Ethylene oxide	Meek *et al.* (2003)
	4-Aminobiphenyl	Cohen *et al.* (2006a)
Mammary tumors associated with suppression of luteinizing hormone	Atrazine	Meek *et al.* (2003)
Thyroid tumors associated with increased clearance of thyroxine	Phenobarbital	Meek *et al.* (2003)
	Thiazopyr	Dellarco *et al.* (2006)
Bladder tumors associated with the formation of urinary tract calculi	Melamine	Meek *et al.* (2003)
Liver/kidney tumors associated with sustained cytotoxicity and regenerative proliferation	Chloroform	Meek *et al.* (2003)
Acute renal toxicity associated with precipitation of oxalate	Ethylene glycol	Seed *et al.* (2005)
Androgen receptor antagonism and developmental effects	Vinclozolin	Seed *et al.* (2005)
Nasal tumors associated with DNA reactivity and cytotoxicity	Formaldehyde	McGregor *et al.* (2006)

from traditional and evolving methods for assessing toxicity. This includes explicit consideration of the comparative weight of evidence and associated uncertainties for several options for hypothesized modes of action early and throughout the analysis. The critical relevance of the kinetic and dynamic information considered in the mode of action analysis for subsequent characterization of dose–response relationships for effects considered relevant to humans (Boobis *et al.*, 2009; Julien *et al.*, 2009), including choice of chemical-specific adjustment factors (Boobis *et al.*, 2008), has also been amplified. Experience in mode of action analysis has also been instructive in contextualizing appropriate application of information from evolving methods of toxicity testing at different levels of biological organization as a basis for more efficient testing strategies.

Both cancer and non-cancer effects are addressed, in recognition that their separation in earlier publications reflected principally evolving experience in mode of action/human relevance analysis rather than variation in conceptual premise. In fact, mode of action analysis facilitates harmonization of cancer and non-cancer assessment. Harmonization in this context refers to a biologically consistent approach to risk assessment for all endpoints, for which exploration of biological linkages is critical to ensuring maximal utility of relevant information. Often, for example, cytotoxicity in an organ is a critical key event that may lead to an increase in cell proliferation and tumors at the same site.

Objectives

This paper has been prepared as an addendum to the previous WHO/IPCS guidance on mode of action/human relevance analysis (Boobis *et al.*, 2006, 2008). While the underlying principles and methodology are similar, the guidance has been updated to reflect recent developments. Some of these developments result from advances in toxicity testing and non-testing methods, and some reflect evolving experience in mode of action/species concordance analysis (additionally referred to herein as mode of action analysis). More detailed information on the nature of systematic hypothesis generation and weight of evidence considerations in mode of action analysis with illustrative case examples is included in the earlier publications referenced in Table 1.

This paper also expands the scope of previous manuscripts to reflect increased understanding of the role of mode of action/species concordance analysis in integrating information from different levels of biological organization. In addition, while early focus of mode of action analysis related to increasing transparency in documenting an operative mode of action with a reasonably high degree of confidence as a basis for risk assessment and regulatory decision-making, the current paper addresses a much broader range of contexts. These include implications for priority setting and testing strategies for both individual chemicals and chemical categories where a less refined analysis and/or higher uncertainty may be acceptable. Summaries of cases selected to illustrate examples of broad application in a research/regulatory context are included here. Readers are referred to the cited documentation for more detailed information on the data analysis for these cases.

Background/Terminology

Mode of action, as previously defined, is a biologically plausible series of key events leading to an effect (Sonich-Mullin *et al.*, 2001). Originally, mode of action was considered principally in the context of late-stage key cellular, biochemical and tissue events. A key event is an empirically observable step or its marker, which is a necessary element of the mode of action critical to the outcome (i.e., necessary, but not necessarily sufficient in its own right); key events are measurable and reproducible. The mode of action framework is based, then, on the premise that any human health effect caused by exposure to an exogenous substance can be described by a series of causally linked biochemical or biological key events that result in a pathological or other disease outcome. (The term mode of action implies no judgment about adversity of effect, though for risk assessment application, the relevant identified or presumed effects are most often considered adverse.) While originally and often simply conceptualized and illustrated as a linear series of key events, in reality, mode of action involves interdependent networks of events with feedback loops. Disease outcomes are initiated or modified within these networks. Differences in networks between and within human and animal populations account, in part, for interspecies differences and human variability.

Early key events in hypothesized modes of action are most often related to chemical characteristics, i.e., those characteristics of structure and/or physicochemical properties that promote interaction of the substance with biological targets. Later key events are less chemical specific and more often an expected consequence of progression of earlier key events (e.g., regenerative proliferation resulting from cytotoxicity).

An adverse outcome pathway is conceptually similar to a mode of action. It was initially described by the computational ecotoxicology community (Ankley *et al.*, 2010) and has been adopted within an international initiative to document, develop and assess the completeness of potentially predictive tools for adverse ecological and human health effects (OECD, 2012). A focus of adverse outcome pathways is on the initial associated chemically mediated "molecular initiating event," equivalent to an early key event in a mode of action.

The terms mode of action and adverse outcome pathway should be interchangeable, representing essentially the subdivision of the pathway between exposure and effect in either individuals or populations into a series of hypothesized key events at different levels of biological organization (e.g., molecular, subcellular, cellular, tissue) (Fig. 1). (The term toxicity pathway, introduced by the US National Research Council in 2007 [NRC, 2007], essentially focuses on a subset of early events leading to an effect at the molecular and cellular levels. These events can be considered critical upstream elements of a more expansive mode of action description of how a chemical can affect human health.) The distinction between mode of action and adverse outcome pathway is artificial, a result principally of experience in the human health versus ecological communities, though it has sometimes been stated incorrectly that, unlike adverse outcome pathway, mode of action does not extend from the individual to the population level. It should be noted, though, that the term mode of action, *per se*, does not imply adversity of outcome. Mode of action, as defined here, could apply equally well to effects that are not adverse, such as therapeutic interventions or health benefits (e.g., from nutritional supplements). Also, focus on human health risk assessment has traditionally been on (often later) key events that provide quantitative information relevant to intraspecies and interspecies extrapolation and life stage susceptibility for dose–response analysis, compared with the molecular initiating event in ecological health assessment. For this reason, considerations relevant to weight of evidence analysis may differ.

Appropriately, given their conceptual similarity, it has been proposed that the weight of evidence for both hypothesized modes of action and adverse outcome pathways should draw upon modified Bradford Hill considerations (Hill, 1965). This proposal was based on a desire to increase transparency and consistency in organizing, linking and integrating information at different levels of biological organization into a more efficient, hypothesis-driven approach to chemical data generation and assessment and use of non-test (e.g., read-across and grouping of chemicals) and *in vitro* methods.

However, there are a number of limitations that remain to be addressed in the proposed reliance on modified Bradford Hill considerations for documentation of mode of action where focus has been on the molecular initiating event (i.e., structure–activity modeling). For example, weight of evidence for hypothesized modes of action in human health risk assessment has traditionally relied heavily on the modified Bradford Hill considerations of concordance of dose–response relationships between key and end events. In addition, influential in mode of action analysis is specificity, which in this context has related to experimental verification that a key event is causal. And while experience in mode of action analyses for documented (adverse) effects in human health risk assessment can inform consideration of weight of evidence for hypothesized modes of action or adverse outcome pathways, based on early key or molecular initiating events, to date, information on dose–response concordance and specificity has not been available in characterizing weight of evidence for hypothesized adverse outcome pathways. This detracts considerably from transparency in documentation of their supporting evidence.

Mode of Action/Adverse Outcome Pathways—Levels of Biological Organization

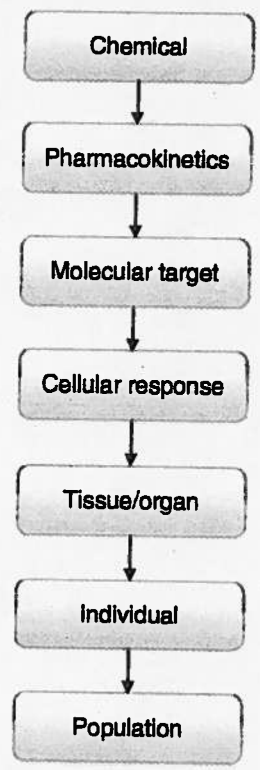


Figure 1. Different levels of biological organization in mode of action analysis. Confidence in an hypothesized mode of action generally increases with increasing evidence at higher levels of biological organization.

Mode of Action Roadmap

There is growing recognition of the need for more efficient methods and strategies to assess the hazards, exposures and risks of the wide array of chemicals to which humans are exposed. This has been reflected in, among others, progressive regulatory mandates in Canada, the European Union and, more recently, the Asian Pacific region to systematically consider priorities for risk management from among all existing chemicals (see, for example, Council of Labor Affairs, Taiwan, 2012; Dellarco *et al.*, 2010; European Commission, 2006; Hughes *et al.*, 2009; Lowell Center for Sustainable Production, 2012; Meek and Armstrong, 2007). This necessitates focus on efficiently prioritized chemicals and endpoints, rather than the traditional time- and resource-intensive series of standard *in vivo* toxicology studies. It also requires the development and integration of information on key events within (hypothesized) modes of action very early in the evaluation process that will enable effective use of data collected from lower levels of biological organization and non-test methods, such as (quantitative) structure–activity relationships ((Q)SAR) and read-across *in vitro* assays.

Figure 2 presents a "mode of action roadmap" to illustrate the iterative process whereby principles and concepts of mode of action analysis can be applied throughout human health risk assessment, with the extent of the analysis being tailored to the issue under consideration. Critical to this more tailored consideration of appropriate testing and assessment strategies is formal, transparent consultation with risk managers, with public accountability, where possible, for the relevant extent of resource investment to address the problem at hand (i.e., problem formulation).

Problem formulation (Fig. 3), the first step in the roadmap (Fig. 2), involves consideration of the risk management scope and goals in relation to relevant exposure scenarios, available resources, urgency of the assessment and the level of uncertainty that is acceptable. This includes consideration of appropriate methods and endpoints for hazard assessment and a mode of action analysis plan tailored to the nature of the decision to be made. For example, decisions concerning chemical prioritization for testing and/or assessment will likely allow for higher levels of uncertainty than those related to establishing regulatory standards. In problem formulation, then, the complexity of the envisaged mode of action analysis is tailored to the context of decision-making; approaches are necessarily flexible and iterative, permitting efficient identification and generation of the essential information to serve as a basis to assess and manage risks appropriately.

The second step in the roadmap (Fig. 2) is to assimilate and consider, in iterative fashion, information on mode of action in the "Modified framework" (see below). This entails hypothesis-based analysis of the weight of evidence for operative key events based on the modified Bradford Hill considerations and qualitative and quantitative concordance of the key events within and between species (Boobis *et al.*, 2006, 2008; Meek *et al.*, 2003; Seed *et al.*, 2005). Early consideration of hypothesis-based key events in the mode of action during problem formulation facilitates incorporation of data from different sources and provides a framework by which it can be organized, integrated and linked at different levels of biological organization (Fig. 3). This includes information generated by evolving methods, such as those targeting cell signaling pathways. The

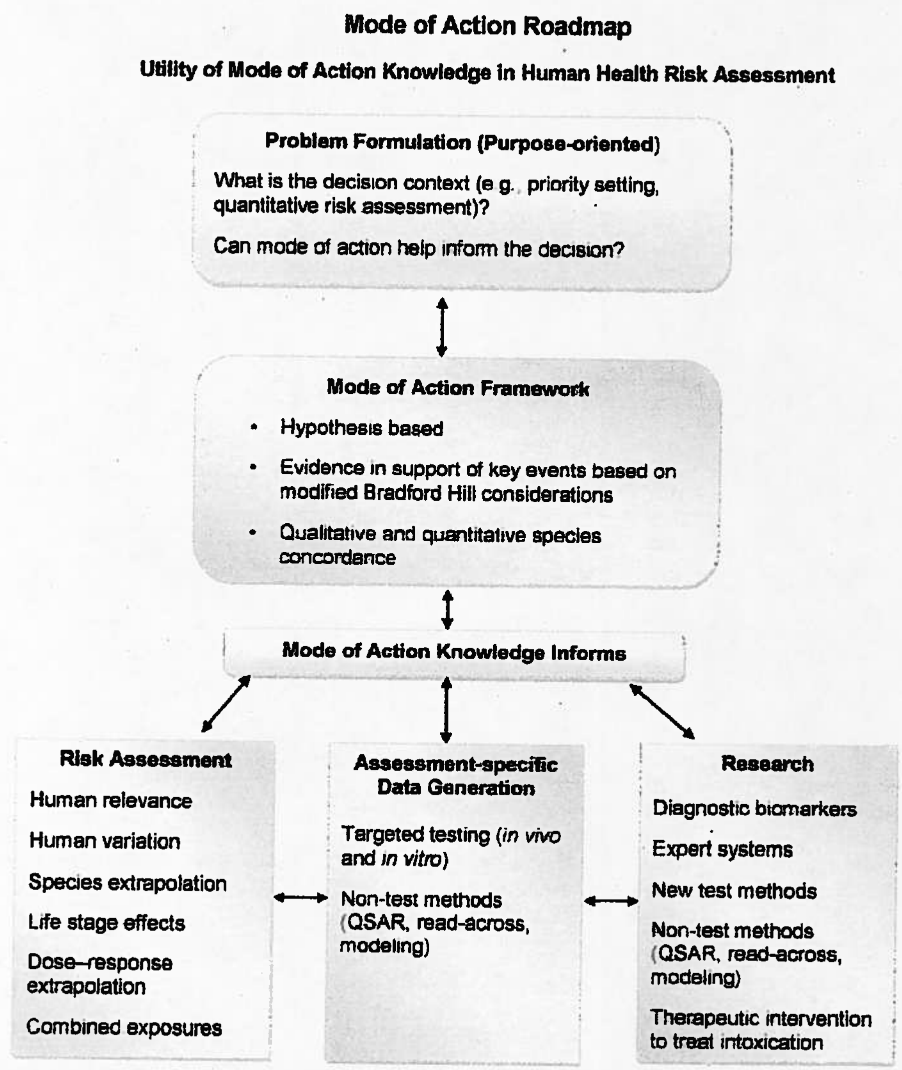


Figure 2. Mode of action roadmap illustrating the use of mode of action knowledge in human health risk assessment. The extent of analysis is tailored to the issue under consideration through iterative analysis and consultation among the assessment, management and research communities.

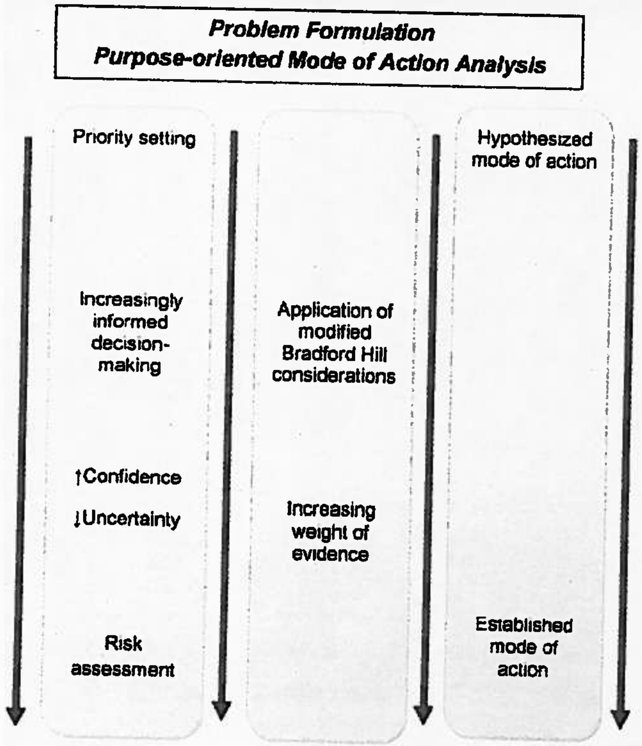


Figure 3. Confidence/uncertainty in "fit for purpose" mode of action/species concordance analysis: correlation of confidence/uncertainty with extent of weight of evidence.

amount of detail and "linearity" characterizing the key events within a hypothesized mode of action can vary as a function of the toxicity of interest, existing knowledge and risk assessment or testing needs.

The mode of action analysis, completed to address the goals outlined during problem formulation, informs one or more of three analytical domains (shown at the bottom of Fig. 2):

(1) risk assessment, including qualitative and quantitative human relevance and variability (e.g., effects at various life stages and within susceptible subgroups), dose–response extrapolation and potential for combined effects of chemicals;
(2) hypothesis-based targeted testing or application of non-test methods to meet the objectives specified in problem formulation, including efficient grouping of chemicals and consideration of read-across, (Q)SAR modeling or appropriate testing within a category approach to fill data needs; and
(3) research priorities relevant to the development of new test and non-test methods, biomarkers and expert systems that feed back to the risk assessment and therapeutic intervention strategies (for intoxication).

As depicted in the roadmap (Fig. 2), mode of action analysis is envisioned as an iterative hypothesis generating and testing process that defines how to assess or test strategically based on risk management needs. As analyses are completed, the problem formulation, testing strategy and risk assessment can be further refined for the decision context.

This iterative process can be illustrated with the following hypothetical example, for which there are considerable data on hazard. While this example draws on a relatively extensive data set, it provides a model for considering significantly fewer data on similar compounds, if they are taken into account from the outset in problem formulation. Initially, a risk manager requests that a risk assessment for the general population be conducted for chemical X, for which exposures of potential concern are those through drinking water. In relatively extensive (traditional) toxicity studies (including a cancer bioassay), chemical X has caused liver tumors in rodents. There is controversy regarding the relevance of this particular tumor type for human health risk assessment, and, based on the preliminary mode of action/species concordance analysis in problem formulation, the risk manager is informed that knowledge of the mode of action of induction of tumors in the relevant dose range could inform conclusions on human relevance. Conduct of appropriate studies to address important data needs and uncertainties in the mode of action analysis can then be considered collectively by the risk manager/risk assessor in a refined problem formulation, depending on resources available and time frame for completion.

If additional generation of data is deemed appropriate, the assessment enters the "research" portion of the roadmap, but with a focused effort on generating data relevant to the mode of action/risk assessment question at hand. The targeted relevant mechanistic data that would inform additional assessment and/or management do not require full knowledge of the mechanism, but rather often quantitative information on determinants of key events, as a basis to predict interspecies differences and human variability better. Upon completion of relevant studies and subsequent mode of action/species concordance analysis, the risk manager is informed of the conclusion (i.e., whether data are considered sufficient to support the hypothesis that the tumors are unlikely to be of relevance to humans).

A potential variant includes the scenario that since the initial problem formulation, the risk manager has become aware that several other related chemicals co-occur with the substance of interest, which may be appropriate for consideration in the same category with chemical X in the risk assessment. The risk manager is informed that the rationale for inclusion of other category members would be strengthened if the same mode of action was suspected; relative potency could then be considered through targeted testing of an early key event. The assessment process now enters the "assessment-specific data generation" portion of the roadmap. Problem formulation can be an iterative process; thus, the results of the targeted testing would further inform the risk manager as to which chemicals within the category are hypothesized to act via the same mode of action, and therefore which should be included for read-across in a combined risk assessment. The assessment process then enters the final "risk assessment" portion of the roadmap.

Modified Framework

The mode of action framework addresses two key questions. The first is whether there are sufficient data to hypothesize, with an acceptable level of confidence, a mode of action for a known or suspected toxicological outcome. The second is the extent to which such a mode of action would, or is likely to, operate in humans at relevant exposure levels (species concordance analysis).

The framework can also be used in two quite different ways, the first reflecting how it was initially developed, for relatively data-rich chemicals. In this case, causal key events related to an observed (adverse) effect associated with a specific chemical exposure are identified as a basis to utilize available data on kinetics and dynamics maximally to inform relevance to humans and subsequent dose–response analysis; this is referenced below as "Application of the mode of action framework for observed (adverse) effects" and reflects historical experience as is illustrated in many of the case studies currently available. Following problem formulation (Figs 2 and 3), then, a decision may be taken that a mode of action analysis would be of value in addressing an observed toxicological response for which the margin between measures of hazard and estimated human exposure is such that it warrants additional refinement of the assessment.

The second way in which the framework can be applied is based on information on key events from appropriate *in vitro* and *in silico* systems to predict and assess potential modes of action and potential consequent (adverse) effects (referenced below as "Application of the mode of action framework in hypothesizing (adverse) effects"). The outcome of such an analysis may be the development of a plausible case to predict an (adverse) effect based on knowledge of putative key events or, alternatively, the probable exclusion of certain (adverse) effects, based on an absence of a likelihood of perturbation leading to relevant key events.

In this context, mode of action comprises a series of causally associated key events leading to, potentially leading to or hypothesized to lead to an (adverse) effect. Hence, there can be only one mode of action for one chemical or group of chemicals leading to a specified effect under a given set of conditions. However, different chemicals, or the same chemical under different conditions (e.g., at higher doses or concentrations), may produce the same effect via different modes of action. An example would be the generation of site of contact tumors in the nasal cavity. One chemical may produce such an effect through cytotoxicity and subsequent cell replication promoting spontaneous mutations, another through DNA reactivity leading to gene mutations promoted by regenerative proliferation secondary to cytotoxicity, and a third through interaction with DNA leading to early mutations. In addition, early key events in competing pathways may, or often, converge to produce the same late key event (and outcome). Each mode of action comprising a series of key events for a given response will be different, but some of the key events may be common to other modes of action leading to the same response. The nature of the key events involved will have an impact on the shape of the dose–response curve and on interspecies and intraspecies differences.

The modified mode of action framework is outlined in Fig. 4 and explained in further detail below.

Application of the Mode of Action Framework for Observed (Adverse) Effects

Only this first approach was addressed in the previous descriptions of the WHO/IPCS/ILSI-RSI mode of action/human relevance framework (Boobis *et al.*, 2006, 2008; Meek *et al.*, 2003; Seed *et al.*, 2005), from which further detailed information can be obtained. Extension of the approach through application to help construct more predictive groupings of chemicals was subsequently highlighted in Carmichael *et al.* (2011). A key aspect of the approach, as illustrated through case studies, is that there should be an unequivocal effect to address before embarking on a mode of action analysis. Hence, problem formulation will

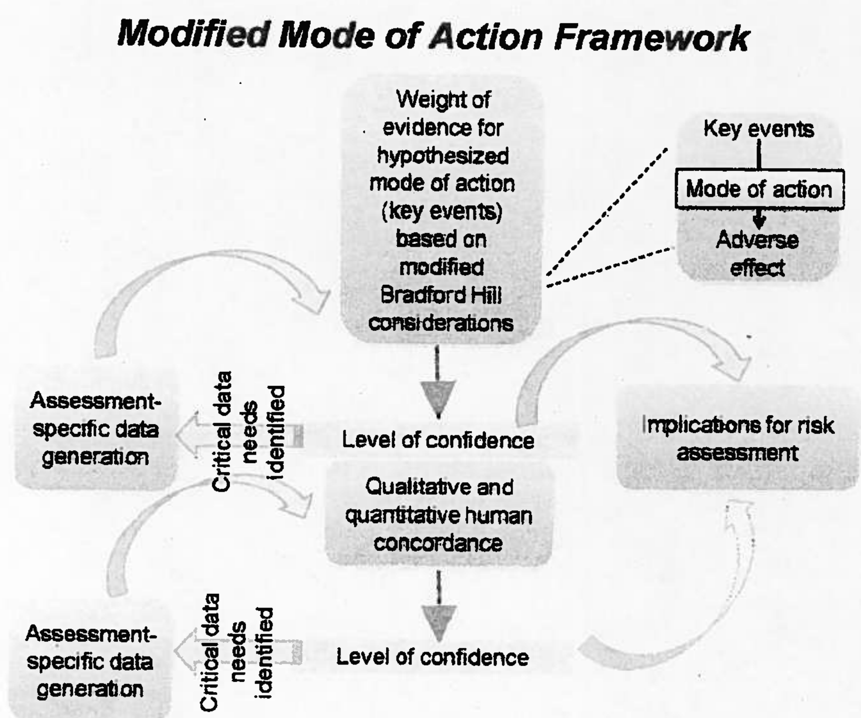


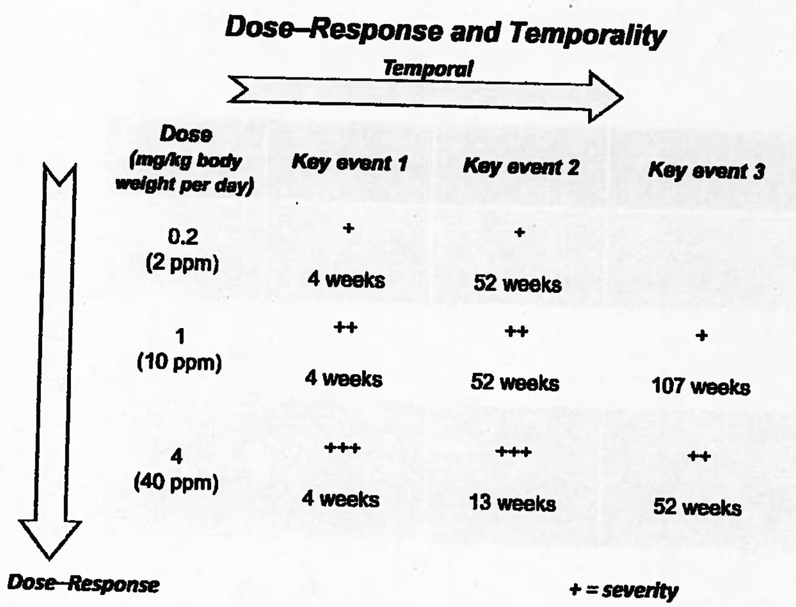
Figure 4. Modified mode of action/human relevance framework and its relation to data needs identified and risk assessment. The application of the framework to assess for observed (adverse) effects and in hypothesizing (adverse) effects is illustrated. The iterative nature of the analysis and the importance of expressing uncertainty are also highlighted.

have identified the (critical) effect(s) of concern to be considered in the analysis.

In general, mode of action analysis applies to a single effect in a single tissue. In essence, there is one mode of action leading to an effect of interest in the relevant organ for a given substance. This mode of action entails several key events, each of which may result from different (sometimes) competing mechanisms and/or pathways, although these converge at a late stage to produce the (adverse) effect. It is important, then, to robustly synthesize available information based on multidisciplinary input in hypothesizing potential modes of action. In addition, in the absence of information to the contrary, site concordance between animals and humans is generally assumed, at least as an initial premise. This is often the case, for example, for many non-genotoxic carcinogens that act through perturbation of physiological processes. Similarly, for many non-cancer

Modified Bradford Hill Considerations

- Concordance of dose–response relationships between key and end events
    - Dose–response relationships for key events would be compared with one another and with those for endpoints of concern
        - Are the key events always observed at doses below or similar to those associated with the toxic outcome?
- Temporal association (time)
    - Key events and adverse outcomes would be evaluated to determine if they occur in expected order



- Consistency and specificity
    - Is the incidence of the toxic effect consistent with that for the key events?
        - i.e., Less than that for the key events?
    - Is the sequence of events reversible if dosing is stopped or a key event prevented?
- Biological plausibility
    - Is the pattern of effects across species/strains/systems consistent with the hypothesized mode of action?
    - Does the hypothesized mode of action make sense based on broader knowledge (e.g., biology, established mode of action)?

Figure 5. An illustration of the modified Bradford Hill considerations for weight of evidence of hypothesized modes of action. The illustration represents evolution of these considerations based on increasing experience in application in case studies and training initiatives internationally. Specific questions being addressed by each of the considerations are offered as a basis potentially to increase common understanding and consistency in their application in mode of action analysis.

Comparative Weight of Evidence for Hypothesized Modes of Action: Cytotoxic Mode of Action
Example Summaries

Modified Bradford Hill consideration	Supporting evidence	Potentially inconsistent evidence
Dose–response Temporal concordance	Metabolism, cytotoxicity, proliferation precede tumors; tumors observed only at cytotoxic doses (benchmark dose analysis) *(qualify based on nature and number of studies)*	Tumors observed at doses lower than those at which key events observed
Consistency, specificity	Consistency in repeated studies and different labs and across species, sexes, routes and levels of biological organizaton *(tls)* correlating with extent of metabolism. No adverse effects without relevant enzyme in null mice. Incidence of tumors less than that for key events and tissue recovery in reversibility studies.	Incidence of tumors greater than that for key events
Biological plausibility	Consistency with state of knowledge on cancer	

Comparative Weight of Evidence for Hypothesized Modes of Action: Mutagenic Mode of Action[a]
Example Summaries

Modified Bradford Hill consideration	Supporting evidence	Potentially inconsistent evidence
Dose–response Temporal concordance	Dose–response and temporal pattern for genotoxicity and tumors consistent with the compound acting via a mutagenic mode of action	Parent compound negative for mutation in a range of *in vitro* and *in vivo* bioassays *(qualify based on nature and number of studies)*
Consistency, specificity	Evidence in a range of well conducted bioassays that mutation is an important early key event (e.g., occurs early and at relevant doses)	The pattern of genotoxicity results inconsistent with what would be expected for the hypothesized mode of action (e.g., not mutagenic in a range of assays; metabolite induces mutation at cytotoxic doses)
Biological plausibility	Pattern of results for genotoxicity consistent with that observed for chemicals known to act via a mutagenic mode of action	Pattern of results for genotoxicity inconsistent with that observed for chemicals known to act via a mutagenic mode of action

[a]Where mutation is an early and influential key event.

Figure 6. An example of comparative weight of evidence for hypothesized cytotoxic and mutagenic modes of action. Information in each of the columns provides an overview of the extent and nature of the available data and its cohesiveness. Particularly important in interpretation of relative weight of evidence is the nature and extent of data that may be inconsistent with a hypothesized mode of action. In this particular case, the extent of inconsistent data is considerably less for a hypothesized mode of action where mutation is likely to be secondary to cytotoxicity than for a mutagenic mode of action (i.e., where mutation is an early and influential key event). Indeed, the pattern of data on genotoxicity is completely consistent with a cytotoxic mode of action. This would lead to the conclusion that there is greater confidence in the chemical acting by a cytotoxic than by a mutagenic mode of action.

Concordance Table with Dose–Response

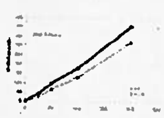
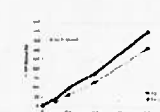
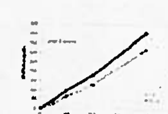
Key event / adverse outcome	Qualitative species concordance	Evidence base	Quantitative species concordance	Quantitative dose–response
Metabolism by cytochrome P450 2E1	Relevant enzyme in kidney and liver of humans	Considerable in animals; limited but relevant to humans	Physiologically based pharmacokinetic model incorporating metabolic rates, enzyme affinities and distribution based on *in vitro* human data supported by *in vivo* data	
Sustained cell damage and repair (cytotoxicity, proliferation)	Liver and kidney target organs in humans	Considerable in animals; possible in humans, but limited data	No data	
Liver and kidney tumors	Possible in humans	Considerable in animals; highly plausible in humans	No data	

Figure 7. An illustration of a concordance table including dose–response curve. The kinetic and dynamic data considered in assessment of mode of action are directly relevant to dose–response analysis, which takes into consideration dose–response relationships for each of the key events.

endpoints, site concordance between test species and humans is a reasonable first assumption, based on considerations of biological plausibility and chemical-specific mechanistic data.

However, there are exceptions to this general principle. Consistent with species- and tissue-specific variation in metabolic activation and detoxification, site concordance for DNA-reactive carcinogens or other effects for which metabolism is critical is often poor. Similarly, for some non-cancer effects induced through a pleiotropic response, such as those that are endocrine mediated, site concordance should not be assumed, but rather considered, based on available mechanistic data and knowledge related to biological plausibility.

These possibilities would need to be scoped at the outset of any mode of action analysis. In such cases, it may be that mode of action analysis would benefit from considering multiple sites in the same evaluation. However, care must be taken to ensure that the mode of action for each effect is likely to be the same, which will not always be the case.

Mode of action analysis relies upon biological plausibility and coherence. The weight of evidence for a hypothesized mode of action is addressed based on the Bradford Hill considerations, proposed originally to examine causality of associations observed in epidemiological studies, but later modified in WHO/IPCS and ILSI-RSI publications on the mode of action/human relevance framework (Boobis *et al.*, 2006, 2008; Meek *et al.*, 2003; Seed *et al.*, 2005) and additionally evolved, here. The original templates for consideration of the weight of evidence for a hypothesized mode of action were based on consideration of traditional measures of toxicity, such as biochemical and histopathological parameters in experimental animals. These templates have been adapted here (Figs 5–7) to reflect additional experience gained in the application of the framework in an appreciable number of case studies over the past decade and as a basis potentially to encompass additional early key events from evolving methods to reliably predict human health outcomes. Based on this experience, robust consideration of dose–response relationships and temporal concordance for early key events will be important in documenting weight of evidence for proposed adverse outcome pathways.

Relevant considerations include dose–response relationships and temporal concordance between specified key events and outcome, consistency (of, for example, the incidence of key events and outcome and changes in causally associated key events), specificity (in the context of essentiality of key events and reversibility) and biological plausibility, based on coherence with the state of knowledge.

In relation to dose–response relationships and temporal concordance, a key event cannot play a role in an (adverse) effect if it is manifest only after toxicity has occurred or if it occurs only at doses higher than those inducing toxicity. The same applies to late key events relative to early key events. There is often a close relationship between dose and time dependency, so that the higher the dose, the earlier a key event is observably affected, and vice versa. This pattern of dose-response and time–response relationships can be invaluable in assessing weight of evidence for a hypothesized mode of action and its key events or how different key events are interrelated. Systematic consideration of dose–response relationships and temporal concordance between key events and (adverse) effects, as illustrated in Fig. 5, encourages early assimilation of relevant information from the broader database of both short- and long-term studies, or from different non-animal test systems, in a mode of action context.

More detailed discussion on all of the modified Bradford Hill considerations when applied in the mode of action analysis for observed (adverse) effects is provided in previous publications on the mode of action/human relevance framework and will not be repeated here. Application and weighting of these considerations continue to evolve as a basis to additionally increase consistency and transparency in assessing weight of evidence in mode of action/species concordance analysis.

It is essential at the outset of mode of action/species concordance analysis that all reasonably plausible modes of action be considered. These include those modes of action that have previously been associated with the relevant effect and any series of key events that logically presents because of available experimental information. The case for each plausible mode of action should be evaluated systematically from the outset, using modified Bradford Hill considerations.

Weight of evidence for alternative hypotheses should be considered and assessed comparatively. Figure 6 illustrates such an evaluation. Based on relative weight of evidence, it can be determined whether one mode of action could be considered with reasonable certainty to explain the (adverse) effect. Where it is not possible to exclude one or more modes of action, critical data needs could be identified as a basis to inform relevant research that could reduce uncertainty concerning the causal key events within a mode of action, depending on the needs and urgency of the assessment as considered in problem formulation.

The degree of confidence in the outcome should be specified, and each step in the mode of action analysis should be accompanied by a list of the critical uncertainties (i.e., lack of knowledge) and associated data needs, prioritized on the basis of their likely impact, if filled, on weight of evidence and implications for subsequent dose–response analysis.

The comparative analysis of weight of evidence for hypothesized modes of action based on the modified Bradford Hill considerations is followed by statements on the likelihood of each being operative to induce the critical effect. Alternatively, depending on the needs and urgency of the assessment addressed in problem formulation, plausible modes of action should be considered as a basis to contrast strengths and weaknesses of different approaches to quantification of interspecies and intraspecies extrapolation in dose–response modeling. This enables risk managers to distinguish best-supported options (i.e., those that are most certain), which is critical in increasing transparency in separating science judgment (i.e., considerations based on experienced consideration of the relevant science base) from science policy determinations (e.g., embedded conservatism in human health risk assessment, incorporated to increase public health protection). Characterization of this nature also contributes to consistency across weight of evidence considerations in different mode of action analyses.

An important objective of framework analysis, then, is the description of the critical sources of uncertainty and characterization of their impact on conclusions concerning weight of evidence for various hypothesized modes of action and their relevance to humans, as a basis particularly for identification of priorities for generation of more or better data. Sensitivity of the estimate to various assumptions can also be tested, and/or available quantitative data relevant to key uncertainties can be analyzed.

Following mode of action analysis and consideration of the associated uncertainties, several outcomes are possible, as illustrated in Fig. 4. There may be sufficient information to conclude that a hypothesized mode of action is supported by available evidence to explain the effect of concern and that key events for this mode of action have been clearly identified. Where there is insufficient information to reach a conclusion, with adequate confidence that a hypothesized mode of action explains the (adverse) effect of concern, appropriate research to address identified critical data needs should provide suitable information to enable confirmation or otherwise of the hypothesized mode of action, through iterative application of the framework. Finally, it may be that at the conclusion of the analysis a hypothesized mode of action is rejected and no other mode of action logically presents itself. In such instances, it may be necessary to proceed with the risk assessment empirically, using relevant information that has been obtained during the analysis of the mode of action – for example, dose–response and time–response information on the endpoint itself, or relevant kinetic and dynamic data.

An important objective of mode of action analysis is to identify those key events that are likely to be most influential in determining potential qualitative and quantitative differences within and between species – that is, key events that are dose and rate limiting. This is addressed in species concordance analysis and is illustrated in Fig. 7. Where it has been possible to conclude that a hypothesized mode of action is adequately supported by the available information with an acceptable level of confidence, it is necessary to consider the extent to which such a mode of action would, or is likely to, operate in humans. Species concordance analysis starts with a statement on the level of confidence in the weight of evidence for the hypothesized mode of action under consideration and associated uncertainties. The extent of this analysis is necessarily dependent upon the test system(s) in which key events have been measured, being less for those that best represent humans.

Consideration of mode of action also enables identification of early events or indicators of susceptibility that could be measured in humans (i.e., biomarkers); for example, if there is sufficient information to support early key events such as metabolic activation to a reactive metabolite, this directs attention to the relevant parameters in humans, as a basis to predict interspecies (based on comparison of the relevant parameters between humans and animals, scaled as appropriate) and intraspecies differences (based on consideration of the relevant parameters within different subgroups of the population). Consideration of potential key events also contributes to identification of any specific subpopulations (e.g., those with genetic predisposition or life stage differences) that may be at increased risk.

Assessment of concordance is accomplished by systematic consideration of the nature of the key events between and within species, taking into account both chemical-specific and more generic information, such as anatomical, physiological and biochemical variations. Concordance is considered both qualitatively and quantitatively (Fig. 7). On rare occasions, it may be possible to conclude that a mode of action identified in studies in animals is not relevant to humans because of profound qualitative differences identified in experimental investigation; for example, the molecular target necessary for a key event is not present in humans, and there is no functional equivalent. An example would be α_{2u}-globulin, which plays a key role in the renal carcinogenicity of D-limonene (see Case example 1) (Meek *et al.*, 2003). Alternatively, and very infrequently, quantitative differences in key events may be so great as to render the mode of action not relevant to humans at any conceivable exposure to the substance.

Case example 1: Lack of human concordance

D-Limonene provides an example of a data-rich case example for which the mode of action has been established with confidence in the animal model and extensive data are available to demonstrate that it is not relevant to humans (Meek *et al.*, 2003).

Hypothesized key events in the mode of action for species- and sex-specific kidney tumors in male rats were the formation of a stable intermediate, D-limonene-1,2-epoxide, which binds to a protein, α_{2u}-globulin, which accumulates in the renal proximal tubule cells, leading to nephropathy and cellular proliferation, and subsequently tumors, at this site following chronic exposure. There is strong evidence that female rats, laboratory mice and other strains of rats for which there is no evidence of D-limonene-related renal toxicity or tumors do not synthesize or express α_{2u}-globulin.

Consideration of the relevance to humans of the key events leading to renal tumors in the male rat model identified the expression of either α_{2u}-globulin or a homologous protein in humans as critical. After an exhaustive analysis, no protein capable of binding to D-limonene-1,2-epoxide could be identified from human kidney, and therefore it could be concluded that the mode of action leading to kidney tumors in the male rat was not likely to be operable in humans.

This is a rare example of a distinct qualitative difference between the animal model and humans, allowing the possibility to conclude that a mode of action is not relevant to humans. However, it is quite unusual to be able to demonstrate such a qualitative difference. Rather, in the vast majority of cases, such differences will be quantitative, and likely differences in sensitivity of response between animals and humans identified in the mode of action analysis would be taken into account in the subsequent dose–response analysis.

If the weight of evidence for the hypothesized mode of action is sufficient and its relevance for risk assessment cannot be excluded, the implications for dose–response analysis and population variability are considered in the context of identified kinetic and dynamic data. Figure 7 indicates the relevance of delineation of key events in hypothesized modes of action considered to operate in humans in subsequent dose–response analysis. In fact, there is a dose–response curve for each of the key events, and risk for the human population is best predicted on the basis of those key events (or a combination thereof) that are likely to be most influential in impacting or preventing risk, taking into account potential interspecies and interindividual differences in kinetics and dynamics as considered in the species concordance analysis. Reliance on earlier key events offers the potential to better characterize and/or acquire data on effects at lower doses or concentrations in human tissues or populations, which are more relevant for risk assessment. It also contributes to the development of more relevant and informative data for human life stages and subpopulations. For Case example 2, these data could be used additionally in quantitative species concordance analysis, with implications for subsequent dose–response analysis, the identification of critical data needs and the contribution of evolving methods – in this case, well-designed genomic studies – see "Application of the mode of action framework in hypothesizing (adverse) effects" below (see also Table 2).

Case example 2: Use of kinetic and dynamic data in species concordance analysis and implications for dose–response analysis – Contribution of well-designed genomic studies

This example illustrates the manner in which kinetic and dynamic data may potentially inform quantitative concordance analysis, including interspecies variation and human variability and, subsequently, dose–response analysis and extrapolation. The example also illustrates how mode of action/species concordance analysis informs meaningful generation of critical data relevant to risk assessment, including that from evolving methods.

Cacodylic acid (dimethylarsinic acid) is a pesticide that causes dose-related increases in the incidence of bladder tumors in rats, but not mice (Cohen *et al.*, 2006b, 2007; US EPA, 2005b). Incidence is increased significantly only at the highest administered dose levels. The parent compound undergoes reductive metabolism to a toxic metabolite, and observed damage to urinary epithelial cells correlates with this pathway (see Cohen *et al.*, 2006b; US EPA, 2005b). The levels of toxic metabolite are significantly increased at doses causing cytotoxicity, proliferative regeneration and bladder tumors. The weight of evidence from critically evaluated data from a wide range of assays both *in vitro* and *in vivo* indicates that the parent compound is not mutagenic, but that the active metabolite is clastogenic at high concentrations or doses. The concentration–response relationships for cytotoxicity associated with the active metabolite were similar in *in vitro* studies in bladder cells of rats and humans. Because of toxicokinetic differences, the toxic metabolite is expected to form at a lesser amount in human urine compared with rats (Cohen *et al.*, 2006b; US EPA, 2005b).

Application of the modified Bradford Hill considerations supported the weight of evidence for the hypothesized key events in the mode of action, which included reductive metabolism and cytotoxicity and proliferative regeneration leading to bladder tumors (Cohen *et al.*, 2006b; US EPA, 2005b). Weight of evidence considerations included a thorough analysis of dose–response relationships and temporal concordance as determined from benchmark dose analyses of a range of *in vivo* studies of different durations. This does not imply a 1: 1 correlation of the incidence of early and late key events (rather, the incidence of early key events is expected to be higher), as key events are essential, but not necessarily sufficient in their own right.

Qualitative and quantitative concordance analysis based on relevant kinetic and dynamic data indicated that these effects are relevant to humans and that quantitative differences would most likely be related to extent of delivery to the target organ of the toxic metabolite and variations in sensitivity of the bladder to damage induced by this metabolite. Chemical-specific adjustment factors could then be derived from a physiologically based pharmacokinetic model incorporating metabolic rates, enzyme affinities and distribution based on *in vitro* human data supported by *in vivo* data and quantitative reflection of the similarity in sensitivity to the active metabolite between the rat and human bladder in *in vitro* studies.

The mode of induction of bladder tumors was deduced principally based on key cytological and biochemical events in mechanistic studies from experiments designed to address critical aspects of both the mode of action and species concordance analysis. The results of genomic studies indicated that similar networks were altered in rat and human urothelial cells exposed to the active metabolite at doses similar to those in urine at which tumors were observed in the critical bioassays. The concordance table in Table 2 outlines confidence/uncertainties in the mode of action/species concordance analysis.

Mode of action analysis also contributes to the interpretation of relatively extensive epidemiological data sets. For example, information on key events in mechanistic studies can contribute to better understanding of expected (not necessarily similar) target organs in humans. This is relevant to the interpretation of negative epidemiological data based on their power to detect the most likely site of damage in humans taking into account mode of action and interspecies differences in key determinants of key events. It also contributes to the selection of appropriate biomarkers of effect in epidemiological studies and to understanding of variations between life stages and subgroups of the human population (see Case example 3).

Case example 3: Role of mode of action analysis in the evaluation of epidemiological data

This case example illustrates the contribution of mode of action analysis when there is substantial human evidence.

Associations between ambient particulate matter exposures and increased cardiovascular mortality were first observed in epidemiological studies without support from animal bioassays, which led to skepticism concerning causality due to the lack of mechanistic underpinning. Subsequent mode of action studies shed light on key events in cardiovascular injury in humans exposed to particulate matter and elucidated interspecies differences and human variability in dosimetry and sensitivity (US EPA, 2009b).

Particulate matter induces adverse effects on the cardiovascular and cerebrovascular systems, such as thrombosis, plaque rupture, myocardial infarction and stroke, via reactive oxygen species, which appear to trigger systemic inflammation through the action of cytokines and other soluble mediators. In general, systemic inflammation is associated with changes in circulating white blood cells, the acute phase response, procoagulation effects, endothelial dysfunction and the development of atherosclerosis. The time course of these responses varies according to the acute or chronic nature of the particulate matter exposure; chronic exposures may also lead to adaptive responses.

If there is appreciable uncertainty about the relevance or applicability of a mode of action, but critical data needs can be identified, it may be possible to obtain such information through conduct of appropriate studies. Table 2 includes the concordance analysis for the example included in Case example 2, illustrating principal areas of uncertainty, where generation of additional data might meaningfully inform the risk assessment.

If it is not possible to establish whether a mode of action would, or is likely to, operate in humans with an acceptable level of confidence, but there is a pressing need for risk management decisions because of the urgency or the nature of the problem, knowledge of dose–response relationships and variability across species may still be of value in later stages of the risk assessment.

The conclusions of the concordance analysis should be accompanied by consideration of associated uncertainty and a statement on the level of confidence that a mode of action would, or is likely to, operate in humans.

Table 2. Concordance analysis of key events in the mode of action associated with induction of bladder tumors in rats by cacodylic acid (Cohen et al., 2006b; US EPA, 2005b)

Key event	Qualitative concordance: Rats	Qualitative concordance: Humans	Quantitative concordance	Confidence/uncertainty
Reduction of cacodylic acid (dimethylarsinic acid, or DMA^{V}) to the highly cytotoxic metabolite, dimethylarsinous acid (DMA^{III}), in urine	Yes: *In vivo* studies detecting DMA^{III} in urine at concentrations that would produce cytotoxicity after DMA^{V} is administered.	Plausible: Evidence following DMA^{V} exposure too limited to draw conclusions, but DMA^{III} shown to be present following human exposure to inorganic arsenic.	Formation of less DMA^{III} in urine of humans compared with rats. Significant levels of additional metabolite trimethylarsine oxide (TMAO) in rodents; detected in humans only at very high doses of inorganic arsenic. DMA^{V} is a poor substrate for the arsenic(III) methyltransferase (AS3MT) in humans. Variation between humans and rats in transport of DMA^{V} across cell membranes. Similar magnitude of response of human and rat epithelial cells to DMA^{III}. Interspecies differences could be taken into account in dose–response analysis through physiologically based pharmacokinetic modeling and use of chemical-specific adjustment factor for dynamics.	Considerable evidence in animals; limited in humans.
Urothelial cytotoxicity	Yes: Scanning electron micrographs of rat urothelium; *in vivo* cytotoxicity findings correlate closely with *in vitro* studies.	Human evidence from *in vitro* studies of urothelial cells, potential to occur *in vivo* in humans if sufficient DMA^{III} is formed.		Considerable consistent evidence that the metabolite leading to urothelial cytotoxicity is DMA^{III} and that cytotoxicity is a rate-limiting key event; quantitative species differences in key events (mode of action) can be taken into account.[a]
Regenerative urothelial proliferation	Yes: *In vivo* 5-bromo-2′-deoxyuridine labeling index data.	No human evidence, but potential to occur in humans if sufficient cell killing is produced and sustained.		Considerable evidence in animals, although some inconsistencies in the data that can be accounted for by variability across different laboratory studies.
Development of urothelial tumors	Yes: Responses in rats but not mice.	No epidemiological data: Only if humans were exposed to doses of DMA^{V} that are sufficiently high to lead to cytotoxic levels of DMA^{III} in the urine.		Strong and consistent evidence supporting the sequence of key events postulated for the development of rat bladder tumors. Good understanding of species differences impacting key events. Evidence in humans is weak. Mode of action is qualitatively plausible in humans, presuming sufficient DMA^{III} is present in the urine.

[a]Though the biochemical target for cytotoxicity is not understood, this information is not essential for the mode of action.

Application of the Mode of Action Framework in Hypothesizing (Adverse) Effects

Lessons learned in mode of action/species concordance analysis for identified effects are also relevant to its application where the (adverse) effect is not demonstrated but could potentially be presumed based on measurement of putative early key events in established modes of action, taking into account lines of available evidence.

Thus, hypotheses about the key events that can lead to the observed (adverse) effect of concern are developed. In contrast, one can also develop hypotheses of potential (adverse) effects that may be triggered by observed putative early key events, based on previous generic knowledge on documented modes of action. Both approaches involve an iterative process of hypothesis testing and data generation.

In this approach, the objective is to identify those modes of action that could plausibly arise from the (series of) key events identified, either because of previous knowledge of their involvement in a mode of action (e.g., for related chemicals for which there are more data) or because a plausible case can be made on the basis of existing biological understanding that such (a series of) events or perturbations may reasonably lead to (adverse) outcomes under certain time- and dose-dependent conditions. The methods used for evaluating putative modes of action will be fit for purpose, which will not necessarily involve one-for-one validation against existing *in vivo* methods. Thus, at the outset, consideration of potential key events in the mode of action plays an integral role both in the choice of experimental methods (*in vivo*, *in vitro* or *ex vivo*) and in data interpretation. Based on the understanding of the causal linkage of putative key events (either observed or anticipated), hypotheses of the likely potential effects of exposure to a chemical are developed in mode of action analysis. Thus, the modified Bradford Hill considerations are just as applicable here, but are not yet well tested.

In terms of quantitative dose–response assessment of the key events, a critical factor is extrapolation of the effect levels *in vitro* or predicted *in silico* to target tissue concentration *in vivo* – for example, by using physiologically based toxicokinetic modeling (referenced as quantitative *in vitro* to *in vivo* extrapolation modeling). Thus, a key consideration is target tissue concentration of the toxicologically active moiety. This approach lends itself well to identification of the causative agent (i.e., parent or metabolite) and readily enables qualitative and quantitative information to be obtained on the enzyme reactions involved. It may be possible to discount human relevance of some putative modes of action based on the margin between effect levels *in vitro* and anticipated target tissue concentrations *in vivo*. This may be particularly important in the short term, when there is substantial uncertainty about the significance of weak signals obtained using *in vitro* methods.

As discussed above, confidence in a mode of action postulated on the basis of putative early key events identified using non-animal methods will depend on the weight of evidence linking these key events with a mode of action for an adverse response from previous studies and on the ability to "calibrate" quantitative changes in the key event against a degree of change known to have adverse consequences. An example would be inhibition of an enzyme involved in neurotransmitter synthesis or degradation. The extent to which this enzyme needs to be inhibited to produce adverse consequences may be known from studies *in vivo* and could then be used to calibrate such changes determined *in vitro* or predicted *in silico*. Integral to this would be knowledge of the extent to which adaptive mechanisms operating *in vivo* are functional *in vitro* or included in the *in silico* model systems.

Formal analysis of site concordance for key events may not be necessary in this approach. Similar to the mode of action analysis for observed (adverse) effects, data may have been generated in tissue-specific model systems or may reflect site-specific key events. Prediction of likely site of effect will require additional considerations, such as the uptake and disposition of the chemical and the activity of causal pathways in different tissues and cell types. For example, if toxicity depends in part upon transport into the target cell to reach a critical concentration, the presence of the transporter in different cell types would be a key consideration in assessing potential site specificity. Similarly, if one of the key events involved inhibition of a specific potassium channel, the tissue distribution of this ion channel would be an important factor in assessing site specificity. Eventually, as knowledge of the biology of the causal pathways increases, it may be possible to use a systems approach to predict likely affected tissues.

Critical to interpretation of data obtained using non-animal methods will be the model system in which information on putative early key events was obtained and whether coverage of more than one key event would be expected. Some key events may be assessed individually (e.g., using *in silico* approaches to predict binding affinity to a receptor), whereas others may be assessed in a more integrated system (e.g., cytotoxicity in a metabolically competent cell system). Alternatively, high-content analysis and bioinformatics may be used to identify those pathways affected by a substance.

In the case of a well-established mode of action, the focus is on determining whether the measured key events provide sufficient evidence to accept the plausibility for the (adverse) outcome without necessarily generating *in vivo* data specifically to demonstrate the (adverse) outcome. Where the mode of action has not previously been established, the possibility that a plausible case can be made because of existing biological understanding should be addressed. Failing this, the likely outcome of such an analysis is the generation of a hypothesis for a possible (adverse) effect, which can then be tested *in vivo*. In any event, once a mode of action is established, the key events are known a priori and can then be assessed *in vitro* or *in silico*. Thus, by understanding the likelihood of effects (i.e., initiation of a toxicity pathway) at lower levels of biological organization (e.g., from SARs and *in vitro* models), it can be determined if more expensive and time-consuming testing at higher levels of biological organization (i.e., *in vivo*) is needed, contributing to increasing efficiency in hazard testing of chemicals. Viewed from the opposite perspective, certain *in vivo* testing could be eliminated for substances that show no potential to initiate the chain of events comprising the mode of action for an (adverse) outcome at environmentally relevant concentrations. In other words, tailored testing can be developed according to screening outcomes indicating the potential for (adverse) effects (see Case example 4).

Case example 4: Use of mode of action analysis to guide development of more efficient testing strategies

Concepts of mode of action analysis are also helpful in guiding developments in the replacement of *in vivo* toxicity testing. Modes of action can be hypothesized based on reference chemicals/pharmaceuticals where the sequence of key events leading to a specific (adverse) effect is known at a sufficient level of detail, as a basis to facilitate identification of the characteristics and requirements of *in vitro* systems and *in silico* models that could predict early and subsequent rate-limiting key events in an integrated manner. Once dose–response relationships between the key events measured *in vitro* and biomarkers of response and ultimately adverse outcome *in vivo* are established for reference chemicals, including the necessary *in vitro* to *in vivo* extrapolation, the toxicity of many other chemicals acting through the same mode of action could in theory be characterized and predicted based on the responses in the *in vitro* systems and *in silico* models.

A large research initiative ("Safety Evaluation Ultimately Replacing Animal Testing," or SEURAT) is based on this premise (Gocht *et al.*, 2013). The first phase of this program, which is co-funded by the European Commission under its Seventh Framework Programme (FP7) and Cosmetics Europe, spans a 5-year period from 2011 to 2015 and includes six research projects, combining the research efforts of over 70 European universities, public research institutes and companies addressing repeated-dose toxicity in hepatic, cardiac, renal, neuronal, muscle and skin tissues. The strategy involves mode of action analysis to describe how any substance may adversely affect human health and to use this knowledge to develop complementary theoretical, computational and experimental (*in vitro*) models that predict quantitative points of departure for safety and risk assessment.

Where data are available on only one or a limited number of key events and the link to an (adverse) effect has not been sufficiently demonstrated, the data may still be of value in helping to rank and prioritize chemicals, as a basis for additional testing and/or decision-making based on likely relative hazard (e.g., relative potency in modulating sodium channels, endocrine disrupting substance prioritization) (see Case example 5).

Case example 5: Mode of action analysis in prioritizing substances for further testing

There is a great deal of interest in prioritizing chemicals for evaluation of endocrine disruption potential (i.e., how best to focus on those chemicals most likely to cause adverse effects without empirically testing all chemicals of regulatory concern). An expert (QSAR) system was developed to predict estrogen receptor binding affinity, using the mode of action (adverse outcome pathway) knowledge (OECD, 2009; Schmieder *et al.*, 2003, 2004; US EPA, 2009a). This pathway is initiated through direct chemical binding to the estrogen receptor, which could plausibly lead to reproductive impairment. The predictive model was developed based on two *in vitro* assays: using a rainbow trout estrogen receptor competitive binding assay to directly measure the chemical–biological interaction and a trout liver slice assay in which the consequences of estrogen receptor activation or inhibition are measurable as a result of tissue uptake and partitioning of the chemical in the presence of xenobiotic metabolism.

More broadly, consideration of SARs for specific key events known to be involved in the mode of action of representative chemicals with the same structural features would be invaluable in helping to construct chemical categories and would enhance the reliability of read-across (see Case example 6 on pyrethroids and Case example 7 on aniline).

Case example 6: Mode of action in the creation of chemical categories

This example addresses the risk assessment of a new synthetic pyrethroid with the same pesticidal mode of action and insecticidal effects as other members of this structural class of compounds. The critical effect of most pyrethroids is reversible neurotoxicity through interaction with a common target, neuronal sodium channels (reviewed in Soderlund, 2012). This mode of action has been established with confidence, and hence the similarity of the pesticidal mode of action of a new member of this chemical group will provide evidence that the compounds share key events. This can be used to support read-across. The risk assessment of a new pyrethroid could then be based on the assumption that it will share a mode of action with other pyrethroids and its likely relative hazard considered in this manner for a first-tier assessment.

The mode of action involves interaction with neuronal sodium channels (Clark and Symington, 2012; Soderlund, 2012). Hence, interaction with sodium channels is a key event for what is often the critical effect. One could rank existing pyrethroids for their potency in modifying the neuronal sodium channel in a suitably designed *in vitro* system and determine the potency of the new compound in this system (Cao *et al.*, 2011b; McConnell *et al.*, 2012). One would also wish to consider basic toxicokinetic aspects, such as absorption (which could be predicted from lipid solubility) (Hou *et al.*, 2009) and metabolic stability (which could be determined in *in vitro* test systems, such as hepatic microsomal fraction or cultured hepatocytes) (Scollon *et al.*, 2009). This information could be used, either semiquantitatively or with a physiologically based toxicokinetic model (Knaak *et al.*, 2012), to inform the choice of reference point from among those of the compounds for which information is already available.

Hence, by using an established mode of action for a structurally well-defined group of compounds with a common toxicophore, it is possible to inform read-across in the early tiers of a risk assessment. This could be refined by evaluating specific key events *in vitro* and using the resulting information to refine the read-across process. In this way, the results of new *in vitro* approaches can be anchored in relevant outcomes by using existing knowledge and concepts.

In addition, such information would help in constructing assessment groups for consideration in the risk assessment of combined exposures to multiple chemicals (Cao *et al.*, 2011a).

Case example 7: Use of mode of action analysis to identify critical data needs and testing strategies in read-across

This case example is based on a case study presented at an Organisation for Economic Co-operation and Development (OECD) workshop held in December 2010. It addresses a mode of action related to the formation of methemoglobin and a number of industrial chemicals that are anilines, which vary in the quantity of toxicity data available (European Chemicals Bureau, 2004). It illustrates how the understanding of the mode of action can focus testing and more effectively fill data needs for data-limited compounds.

Aniline induces methemoglobinemia, which, if severe, can result in hemolytic anemia. Hemolytic anemia is a late consequence of methemoglobinemia and a response to the elimination of circulating red blood cells that contain methemoglobin. Aniline is first metabolized in the liver (probably by cytochrome P450 enzymes) to phenylhydroxylamine. It is further oxidized in red cells, most likely to free radical species, via nitrosobenzene. The iron in hemoglobin is oxidized by the free radical species from Fe^{2+} to Fe^{3+}, in which state (i.e., methemoglobin) it cannot bind oxygen. Decreased oxygen results in hypoxia-induced necrosis in tissues that have high oxygen needs. Damaged red blood cells are sequestered by the spleen and are phagocytosed by splenic macrophages, leading to increased red blood cell production by the blood-forming organs, primarily the bone marrow. If the bone marrow cannot keep up with the replacement needs, then extramedullary hematopoiesis occurs as a compensatory response. To determine the potential of the untested anilines to result in hemolytic anemia, *in vitro* testing could be conducted to measure the formation of phenylhydroxylamine and/or methemoglobin.

Thus, the mode of action framework provides a conceptual construct to consider key events at different levels of biological organization plausibly linked to an *in vivo* endpoint of regulatory interest. This allows for the development and use of alternative (*in vitro*) assays to target particular cellular or physiological key events along a specific pathway. Once the mode of action has been established, the key event data can be used for read-across from other chemicals. If a new chemical fits the established mode of action, this existing knowledge can be used to justify a more efficient testing strategy, so not every chemical needs to be evaluated in an *in vivo* test.

Information on mode of action, or on critical key events, can also be invaluable in helping to construct assessment groups for conducting a risk assessment of combined exposure to multiple chemicals (Meek *et al.*, 2011; see Case example 6).

One conclusion from the application of the mode of action framework to information obtained using non-animal methods could be that the data are sufficiently robust to support an established mode of action with a known causal relationship to an (adverse) outcome. Alternatively, it may be possible to conclude that whereas information on one or more key events is missing, provision of information on this data gap would enable a putative mode of action to be assessed with confidence. Finally, the available data may be such that it is not possible to postulate any mode of action with an acceptable degree of confidence.

Increasing numbers of data warehouses comprising substantial amounts of curated information on interspecies and interindividual variability in parameters relevant to many key events are becoming available. These warehouses cover a wide range of species- and individual-specific information, including human demographics, anatomical, physiological, biochemical, clinical chemical and life stage-dependent parameters, genetic, genomic, epigenetic, transcriptomic, proteomic and metabolomic information, phenotypic variation in cellular and physiological functions, and expression levels and activities of enzymes and transporters of xenobiotic disposition. Such information, together with evolving bioinformatics and computational tools, may facilitate quantitative (both deterministic and probabilistic) analyses of variability and more robust uncertainty analyses. These tools may also enable more effective analysis of the frequency with which alterations of key events and pathways are reported in similar studies, within and across animal species, and among humans. Similarly, they may permit a more thorough analysis of dose, exposure durations and response relationships in pathways across studies.

It should be noted that the availability of larger quantities of data on early potential key events to inform mode of action analyses might lend itself to probabilistic assessments and more robust uncertainty analyses.

Discussion and Conclusions

The WHO/IPCS mode of action/human relevance framework has been updated to reflect experience acquired in its application, as well as extending its utility to emerging areas in toxicity testing and non-testing methods. The underlying principles have not changed, but the scope of the framework has been extended to integrate information at different levels of biological organization and to reflect evolving experience in a much broader range of potential applications. These applications are relevant not only to full risk assessment for individual chemicals, but also to evolving methods for priority setting and assessment to meet increasing demands to more efficiently and accurately assess and manage large numbers of substances. They include read-across and assessment of groups of chemicals and combined exposures. The mode of action/species concordance analysis also informs hypothesis-based data generation and research priorities in support of risk assessment, related not only to (adverse) effects but also to therapeutic intervention strategies.

Envisaged broader application is illustrated in an integrative and iterative roadmap to address needs for assessment identified in formal problem formulation, as a basis to tailor the appropriate extent of mode of action/species concordance analysis. The roadmap, problem formulation and framework are iterative in nature, with feedback loops encouraging continuous refinement of fit for purpose testing strategies and risk assessment.

The relationship between mode of action and the more recently defined "adverse outcome pathway" is also clarified: conceptually, the terms are synonymous, with both representing division of the path between exposure and effect into a series of key events (including early molecular initiating events) for both individuals and populations. However, mode of action does not necessarily imply adversity of effect, as is seemingly implied by the descriptor adverse outcome pathway.

Broader application of the modified mode of action framework is considered in two contexts, including one for which it was originally developed, where the toxicological effects of chemical exposure are known (i.e., when, as a result of problem formulation, there is a desire to perform a mode of action/species concordance analysis for an observed toxicological effect). The outcome of mode of action analysis in this application is acceptance or rejection of a hypothesized mode of action or recommendation for additional targeted research. Various case examples included here illustrate the nature of information required to demonstrate lack of human concordance, the implications of kinetic and dynamic data considered in mode of action analysis for subsequent dose–response analysis and for the design of targeted research studies using new methods (e.g., genomic technologies) and the integration of toxicological and epidemiological data.

The modified framework can also be applied in hypothesizing effects resulting from exposure to a chemical – that is, with information on putative key events in established modes of action from appropriate *in vitro* or *in silico* systems and other lines of evidence to predict and assess the likelihood of a potential mode of action and consequent effects. With the increasing amount of data available from evolving technologies, such as high-throughput and high-content screening assays, QSARs and other computational approaches, it is likely that this latter application of the framework will be of increasing value to the risk assessment community. The considerable experience acquired in the application of the framework in addressing documented (adverse) effects has a meaningful implication to inform the more limited knowledge base in these more predictive applications. This is illustrated in various case examples, including the use of mode of action analysis in prioritizing substances for further testing, in guiding development of more efficient testing strategies and in identifying critical data needs and testing strategies in read-across. In this vein, mode of action considerations should inform further development of research strategies and data generation methods, as well as the development of biomarkers.

The modified Bradford Hill considerations incorporated in framework analysis from its inception are considered a critical element to document, transparently and consistently, weight of evidence for hypothesized modes of action. These considerations have been updated and additionally articulated somewhat here to reflect increasing experience in application for cases where the toxicological outcome of chemical exposure is known. Additional work is also under way to further simplify and delineate application of the modified Bradford Hill considerations in mode of action analysis. This includes additional articulation of the modified Bradford Hill considerations for weight of evidence as a basis to contribute to common understanding, rank ordering of their importance as well as provision of examples of what might constitute strong versus weak evidence for each, based on acquired experience in mode of action analysis (Meek ME, Palermo CM, Bachman AM, North CM, Lewis RJ, submitted).

A template for extension of the concordance table in the original framework to dose–response analysis is also included, as is one for comparative consideration of weight of evidence for various modes of action based on the modified Bradford Hill considerations. Clear and transparent documentation of uncertainties at each stage of the mode of action analysis is also emphasized, with the objective of being as quantitative as possible regarding the likelihood of a hypothesized mode of action being operative in humans. Additional work to delineate more specifically the appropriate form and content of uncertainty analysis is strongly recommended, consistent with objectives and content of ongoing initiatives in this area.

Experience in mode of action analyses for documented (adverse) effects in human health risk assessment is informative in consideration of weight of evidence for hypothesized effects (referenced as adverse outcome pathways by OECD, 2012), based on early key or molecular initiating events. Based on this experience, development of proof of concept for application of the modified Bradford Hill considerations in more predictive application is strongly recommended. This is particularly important, in view of their significant reliance on demonstration of the essentiality of key events and concordance of dose–response relationships and temporality between early and late key events, information that is often lacking in the more predictive application that is envisaged. Additional collaboration between the health risk and ecological communities in this context is also recommended as a basis to draw on collective experience to increase common understanding and to develop communication and uptake strategies.

In conclusion, the modified framework and accompanying roadmap and case examples are expected to contribute to improving transparency in explicitly addressing weight of evidence considerations in mode of action and species concordance analyses based on both conventional data sources and evolving methods. The broader application envisaged here emphasizes the importance of interaction among the risk assessment, risk management and research communities, as a basis to transition to consideration of data from different levels of biological organization in fit for purpose mode of action analysis (e.g., prioritization vs. full assessment), while also highlighting the need to anchor data from evolving technologies and research. Development of the modified mode of action framework has also highlighted the conceptually identical mode of action and adverse outcome pathway and the resulting need for the research and environmental and human health risk assessment communities to move forward together to develop rigorous, efficient and transparent methodologies to meet increasingly progressive mandates to test and assess, more efficiently and more effectively, much larger numbers of chemical substances in commerce.

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Conflict of Interest

The authors did not report any conflicts of interest.

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Review Article

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Mode of action human relevance (species concordance) framework: Evolution of the Bradford Hill considerations and comparative analysis of weight of evidence

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ABSTRACT: The mode of action human relevance (MOA/HR) framework increases transparency in systematically considering data on MOA for end (adverse) effects and their relevance to humans. This framework continues to evolve as experience increases in its application. Though the MOA/HR framework is not designed to address the question of "how much information is enough" to support a hypothesized MOA in animals or its relevance to humans, its organizing construct has potential value in considering relative weight of evidence (WOE) among different cases and hypothesized MOA(s). This context is explored based on MOA analyses in published assessments to illustrate the relative extent of supporting data and their implications for dose–response analysis and involved comparisons for chemical assessments on trichloropropane, and carbon tetrachloride with several hypothesized MOA(s) for cancer. The WOE for each hypothesized MOA was summarized in narrative tables based on comparison and contrast of the extent and nature of the supporting database versus potentially inconsistent or missing information. The comparison was based on evolved Bradford Hill considerations rank ordered to reflect their relative contribution to WOE determinations of MOA taking into account increasing experience in their application internationally. This clarification of considerations for WOE determinations as a basis for comparative analysis is anticipated to contribute to increasing consistency in the application of MOA/HR analysis and potentially, transparency in separating science judgment from public policy considerations in regulatory risk assessment. Copyright © 2014. The Authors. Journal of Applied Toxicology Published by John Wiley & Sons Ltd.

Keywords: human relevance framework; mode of action; weight of evidence; key events; evolved Bradford Hill considerations

Introduction

The mode of action/human relevance (MOA/HR) framework is an analytical framework designed to increase transparency in the systematic consideration of the weight of evidence (WOE) of hypothesized MOA(s) for critical effects and their relevance to humans. It was developed in initiatives of the International Life Sciences Institute Risk Sciences Institute (ILSI RSI) and the International Programme on Chemical Safety (IPCS) and derives from earlier work on MOA by the US Environmental Protection Agency (USEPA) and IPCS (Sonich-Mullin *et al.*, 2001).

The development and evolution of the IPCS ILSI RSI MOA/HR framework, which has involved large numbers of scientists internationally, is described in several publications (Boobis *et al.*, 2006, 2008; Meek, 2008; Meek *et al.*, 2003; Seed *et al.*, 2005). Potential application in a broader range of relevant contexts has been considered more recently (Carmichael *et al.*, 2011; Meek and Klaunig, 2010). The framework has been illustrated by an increasing number of case studies ($n = 30$, currently), and is widely adopted in international and national guidance and assessments (Meek *et al.*, 2008), including those of the USEPA (Dellarco and Baetcke, 2005; Manibusan *et al.*, 2007; SAB, 1999, 2007; SAP, 2000; USEPA, 2005a). Building on this collective experience, the framework has been updated recently, to address uncertainty additionally and to extend its utility to emerging areas in toxicity testing and non-testing methods. The update includes incorporation within a roadmap, encouraging continuous refinement of fit-for-purpose testing strategies and risk assessment (Meek *et al.*, 2014).

In addition to increasing transparency through structured articulation of the evidence and uncertainties upon which conclusions are based, MOA/HR analysis also contributes to the transparent assimilation of all available data in both a risk assessment and research context. This is important because it facilitates identification of critical data needs and contributes to transparency in the separation of science judgment (i.e., weighting of options based on systematic consideration of available scientific support) from public health protection policy, the latter

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sometimes involving embedded conservatism to increase public health protection.

Though the MOA/HR framework is not designed to address the question of "how much information is enough" to support a hypothesized MOA in animals or its relevance to humans, its organizing construct has value in considering relative WOE among different cases and hypothesized MOAs. Comparative WOE evaluation for MOA/HR analysis is illustrated as a basis to increase common understanding of the nature of transparency required to document the relative degree of confidence in supporting data for hypothesized MOAs. To demonstrate this approach, WOE for MOA/HR analysis in two published assessments (i.e., carbon tetrachloride and 1,2,3-trichloropropane [TCP]) (USEPA, 2009, 2010) is comparatively considered in the context of evolved Bradford Hill (B/H) considerations introduced here to promote better common understanding and consistency in use. The focus here is not on the conclusions of the assessments but rather, the utility of comparative analysis for WOE evaluation in MOA/HR analysis. These cases were specifically selected to exemplify varying degrees of WOE for several hypothesized MOA.

Methods And Results

Details of the updated MOA/HR framework are available elsewhere (Meek *et al.*, 2014). Briefly, the WOE for a hypothesized MOA in animals is assessed based on considerations modified from those proposed by Bradford Hill (Hill, 1965) for assessment of causality in epidemiological studies. HR or species concordance is then systematically considered, taking into account more generic information such as anatomical, physiological and biochemical variations. If the WOE for the hypothesized MOA is sufficient and relevant to humans, implications for dose–response in humans are then considered in the context of kinetic and dynamic data. Delineation of the degree of confidence in the WOE for hypothesized MOAs is critical, as is the delination of critical research needs.

Establishing support for or rejection of a hypothesized MOA provides the foundation for subsequent considerations of dose–response, HR and estimates of risk. It involves (1) delineation of key events leading to the end (adverse) effect in a hypothesized MOA and (2) evaluation of all of the data to consider the extent of the supporting WOE for the hypothesized MOA. Importantly, if alternative MOA(s) are supported, these are evaluated with equal rigor in separate MOA/HR framework analyses. Ultimately, depending upon the application, there may be a need to draw a conclusion on the sufficiency of data supporting a MOA, to assess different risk management options. The comparative analysis of WOE was developed as a basis for increasing common understanding of the nature of transparency required to document the degree of confidence in the sufficiency of supporting data for hypothesized (potentially competing) MOAs.

A template for WOE analysis of MOA based on the evolved B/H considerations is presented in Table 1. In this approach, supporting data, inconsistent data and missing information are evaluated and tabulated in the context of the evolved B/H considerations presented here. The data in this table are considered in totality to assess the WOE for a MOA. In addition, the evidence can be used in a comparative manner to gain perspective on the relative degree of confidence that a hypothesized MOA is operative, based on the extent of supporting WOE compared to that for another postulated MOA for the same chemical or for the same MOA for other chemicals.

As illustrated in Table 1, WOE analysis is heavily dependent on the B/H considerations. Previous iterations of modified B/H considerations have been applied inconsistently in MOA/HR analyses, which may be attributable in large measure to the availability of only relatively general, early guidance in this area (USEPA, 2005b; Sonich-Mullin *et al.*, 2001). Some of the considerations have been misinterpreted due to a lack of common understanding of their appropriate level of application to MOA data in a WOE context; i.e., in overall data synthesis and evaluation of sufficiency of evidence to support a MOA decision versus the initial phase of systematic review (i.e., data selection and individual study review). Table 2 summarizes the variation in definitions of the B/H considerations in MOA analysis, which may also have contributed to inconsistency in application.

Evolved B/H considerations have been proposed and clarified here through delineation of the specific aspects addressed by each, as framed by a series of questions (captured below and summarized in Table 3). These questions build on those presented in Meek *et al.* (2014), based on additional experience in considering transparency in existing assessments as a basis to document comparative WOE. These evolved B/H considerations are proposed, then, not only as a basis to increase consistency in making WOE determinations for hypothesized MOA(s), but also to

Table 1. Template for weight of evidence based on evolved Bradford Hill considerations

Evolved Bradford Hill Considerations		Supporting Data	Inconsistent Data	Missing Data
1. Biological Concordance				
2. Essentiality of Key events				
3. Concordance of Empirical Observations among Key Events	Dose–response Temporality Incidence			
4. Consistency				
5. Analogy				

For a postulated mode of action, supporting data, inconsistent data and missing data are tabulated in the context of the evolved Bradford Hill considerations. Input in the supporting and inconsistent columns captures only what has been observed. Input in the missing column includes only that which is technically feasible and that is important for informing the mode of action. Cells are left blank in instances where data do not exist or are inadequate for evaluation. A brief narrative should accompany this table to describe the overall determination as to whether the data support or refute the hypothesis.

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Table 2. Definition of the Bradford Hill considerations for application in mode of action analysis

Bradford Hill Considerations (Hill, 1965)	IPCS MOA/HR Framework (Boobis *et al.*, 2006; 2008; Sonich-Mullin *et al.*, 2001)	EPA Cancer Guidelines (USEPA, 2005b)	Evolved Bradford Hill considerations
Strength Strength of the association between suspected cause and observation.	**Strength** Unclearly defined. Considered together with specificity and consistency.	**Strength** The finding of large risks increases confi dence the association is not due to chance.	**N/A** Not considered applicable for evaluating MOA data.
Consistency Repeatability of an association by different persons, in different places, circumstances and times.	**Consistency** Repeatability of the key events in different studies. Considered together with strength and specificity.	**Consistency** Pattern of elevated risk observed across several independent studies.	**Consistency** Is the pattern of effects across species/ strains/ organs/test systems what would be expected?
Specificity The association is limited to a specific population and to particular sites and types of disease.	**Specificity** Stop/recovery studies show an absence or reduction of toxicity when a key event is blocked or reduced. Considered together with strength and consistency.	**Specificity** One cause associated with a single effect or disease.	**Essentiality of key events** Is the sequence of events reversible if dosing is stopped or a key event prevented?
Temporality The exposure occurs before the effect.	**Temporal association** Key events should be observable before toxicity is apparent.	**Temporal relationship** When exposure is known to precede development of the disease.	**Temporal concordance** Are the key events observed in hypothesized order?
Biological gradient Risk of disease increases with increasing exposure.	**Dose–response relationship** The dose–response for key events parallel the dose–response for the toxic effect. Increases in incidence of a key event correlate with increase in incidence of later key events.	**Biological gradient** Increasing effects associated with greater exposure.	**Dose–response concordance** Are the key events observed at doses below or similar to those associated with the end (adverse) effect?
Plausibility Biological knowledge supports suspected causation.	**Biological plausibility and coherence** Consistent with current understanding of biology. Considered together with coherence.	**Biological plausibility** Consistency with data from experiments or other sources demonstrating biological plausibility.	**Biological concordance** Does the hypothesized MOA conflict with broader biological knowledge? How well established is the MOA in the wider biological database?
Coherence The association agrees with the generally known facts of the history and biology of the disease.	**Coherence** Consistency with what is known specifically about the overall biological effects of the substance. Considered together with biological plausibility.	**Coherence** Information supporting cause and effect from other lines of evidence (i.e., animal bioassays, toxicokinetic studies and short-term studies).	**N/A** Not considered applicable for evaluating MOA data
Experiment Experimental evidence alters the frequency of associated events.	**N/A** Has not been mentioned in recent publications on the MOA/HR framework.	**Experimental evidence** when a change of exposure in a human popu lation brings about a change in disease.	**N/A** Not considered applicable for evaluating MOA data.

(Continues)

Table 2. *(Continued)*

Bradford Hill Considerations (Hill, 1965)	IPCS MOA/HR Framework (Boobis *et al.*, 2006; 2008; Sonich-Mullin *et al.*, 2001)	EPA Cancer Guidelines (USEPA, 2005b)	Evolved Bradford Hill considerations
Analogy Information for a similar but different association supports causation.	**N/A** Has not been mentioned in recent publications on the HR/MOA framework.	**Analogy** Insight gained from structure activity relationships and information on structural analogues.	**Analogy** Would the MOA be anticipated based on broader chemical specific knowledge?
N/A	**N/A** Considered as part of dose–response relationship definition.	**N/A**	**Incidence concordance** Is the occurrence of the end (adverse) effect less than that for preceding key events?

HR, human relevance; MOA, mode of action.

promote consistency in their application based on accumulating experience internationally.

The evolved B/H considerations are described in more detail below. These considerations appear in rank order based on their appropriate weighting of relative contribution to WOE determinations for hypothesized MOA(s), with those listed first contributing most significantly. Examples for evaluating weak to strong evidence for each evolved B/H consideration are also discussed.

Biological Concordance

- Does the hypothesized MOA conflict with broader biological knowledge?
- How well established is the MOA?

Evidence for a hypothesized MOA must satisfy the consideration of biological concordance. If available data on the hypothesized MOA are at odds with biological understanding, the hypothesis does not constitute a reasonable option for consideration. For instance, if a hypothesized early key event cannot conceivably lead to a subsequent hypothesized key or end event, it need not be considered.

The extent of evidence for biological concordance would be considered stronger, for example, if the hypothesized MOA has been well documented for a broad range of chemicals, and weaker if the hypothesized MOA is conceivable based on limited data or it has been hypothesized based simply on the possibility that none of the key events are at odds with biological understanding.

Essentiality of Key Events

- Is the sequence of events reversible if dosing is stopped or a key event prevented (i.e., counterfactual evidence)?

The extent of counterfactual evidence (i.e., experimental support for the necessity of a key event) is one of the principal determinants of WOE for a hypothesized MOA (Borgert *et al.*, 2011). For example, experimental evidence in animal models that lack a key metabolic pathway (e.g., knock out animal models) and fail to develop the end (adverse) effect would support essentiality of a key event. Similarly, if following cessation of repeated exposure for various periods, effects are reversible (i.e., late key events and/or the end (adverse) effect is prevented), this constitutes relatively strong evidence that key events are causal.

It is important to note that by its nature, counterfactual evidence typically addresses the necessity of an individual key event in a hypothesized MOA. Therefore, it may not always be helpful for discerning between two possible MOAs that share a key event. For example, if a chemical requires metabolic activation to be carcinogenic, a negative result in a 2-year cancer bioassay in an animal model null for the necessary activating enzyme supports that metabolism is necessary for carcinogenesis but is not helpful for differentiating between a MOA involving metabolic activation followed by direct DNA damage versus a MOA involving metabolic activation followed by cytotoxicity and regenerative proliferation.

Support for the essentiality of key events is considered stronger when there is direct counterfactual evidence supporting multiple key events in the hypothesized MOA. Evidence is considered weaker when evidence involves indirect measures for key events (i.e., the key event is inferred from the actual measured endpoint)

Table 3. Proposed changes to the Bradford Hill considerations and guidance for interpretation to improve application in the MOA/HR framework[a]

Evolved Bradford Hill considerations	Defining questions	Evidence for evaluating degree of support for the mode of action: Stronger	Evidence for evaluating degree of support for the mode of action: Weaker
1. Biological Concordance (replaces biological plausibility & coherence)	Does the hypothesized MOA conflict with broader biological knowledge? How well established is the MOA?	MOA is well established in scientific knowledge and/or completely consistent with established biological understanding.	MOA is contrary to well established biological understanding. MOA requires biological processes that are novel or poorly established.
2. Essentiality of Key Events (replaces strength, and specificity)	Is the sequence of events reversible if dosing is stopped or a key event prevented?	Counterfactual evidence to support key events (e.g., absence/reduction of later events when an earlier key event is blocked or diminished).	Data on reversibility only, indirect evidence only for key events or limited data available to assess.
3. Concordance of Empirical Observations among Key events (encompasses dose response and temporal concordance and beyond)	Dose–response: Are the key events observed at doses below or similar to those associated with end (adverse) effect? Temporality: Are the key events observed in hypothesized order? Incidence: Is the occurrence of the end (adverse) effect less than that for the preceding key events?	Dose–response and temporality: expected pattern of temporal and dose–response relationships based on robust database (multiple studies with examination of key events at interim time periods and at least 3 doses). Incidence: incidence of early key events is greater than end (adverse) effect.	All key events occur at all dose levels and all time points and/or limited data available to assess (e.g., inadequate dose spacing, missing key time periods for effect development, or failure to assess incidence at early time points). Incidence of early key events is lower than the end (adverse) effect and/or limited data available to assess.
4. Consistency (among different biological contexts)	Is the pattern of observations across species/strains/organs/test systems what would be expected based on the hypothesized MOA?	Pattern of effects are what would be expected across species, strains, organs and/or test systems.	Significantly inconsistent pattern of effects or limited data available to assess (e.g., effect only observed in a single rat strain).
5. Analogy (consistency across chemicals)	Would the MOA be anticipated based on broader chemical specific knowledge (e.g., the chemical is a member of a category for which related chemicals have known or strongly suspected MOA)?	Observations are consistent with those for other (related) chemicals having well defined MOA.	Pattern of effects for other (related) chemicals is distinctly different. Insufficient data to evaluate whether chemical behaves like related chemicals with similar proposed MOA.

MOA, mode of action.

[a]Evolution of the Bradford Hill (B/H) considerations for improved fit-for-purpose in the evaluation of sufficiency of data to support a hypothesized MOA. The evolved B/H considerations are rank ordered based on their appropriate weighting of relative contribution to weight of evidence determinations for hypothesized MOA(s), with those listed at the top contributing most significantly.

or non-specific inhibition of key events. For example, for a MOA hypothesized to involve binding to a receptor, demonstrating an end (adverse) effect is prevented by knocking-out or downregulating expression of the receptor is stronger than counterfactual evidence using a non-specific inhibitor.

Concordance of Empirical Observation Among Key Events

Concordance of empirical observations contributes considerably to the WOE for hypothesized MOA(s). Specifically, concordance of dose–response, temporality and incidence are key considerations. Each of these is addressed separately below. While not weighted as heavily as biological concordance and essentiality of key events, concordance of empirical observation across dose–response, temporality and incidence contributes significantly to WOE. Relationships and outliers should be carefully evaluated to understand whether the WOE strongly supports or is discordant with the hypothesized MOA, including consideration of cohesiveness across all three aspects of empirical observation.

Concordance of Dose–response Relationships Among Key Events

- Are the key events observed at doses below or similar to those associated with the end (adverse) effect?

In past MOA analyses, assessment of dose–response has sometimes been misinterpreted as simply addressing the question: "Is there evidence of a dose–response relationship for key events and/or the end (adverse) effect?" While this question is relevant to hazard characterization, it does not address dose–response concordance in relation to the WOE for a hypothesized MOA. Rather, the latter addresses the consistency of observed dose–response relationships among key and end (adverse) effects, as framed explicitly in the question above.

The hypothesized MOA is not supported in scenarios for which there is evidence that early key events occur only at higher doses than the end (adverse) effect. For example, a hypothesized receptor-based MOA is not supported by evidence indicating that receptor binding occurs only at doses well above those that cause frank liver injury, though it is important to consider if this might be a function of dose spacing in the relevant studies. Benchmark dose analyses for the dose–response relationships in key and end events are the most appropriate measure for consideration of their concordance, as they provide for direct comparison of comparable doses associated with a specified increase in each of the key events and/or end (adverse) effects and normalize for variations in dose spacing and group sizes in different studies.

Examination of the pattern of dose–response relationships is particularly important in considering the degree of support for hypothesized mutagenic MOAs (i.e., where mutation is an early and influential key event). For example, observation of a mutagenic response at high (cytotoxic) doses in genotoxicity assays is supportive of hypothesized MOAs where mutation is a secondary consequence of increased proliferative response resulting from tissue damage.

Concordance of Temporality (Time) Among Key Events

- Are the key events observed in hypothesized order?

Temporal concordance refers to the observation of key events in sequential order as described in the hypothesized MOA. In other words, earlier key events should be observed to precede later key events and the late (adverse) effect. Stronger evidence for temporal concordance is obtained when key events at interim time points demonstrate the hypothesized order (either in a single robust study or across multiple studies). Such evidence can often be acquired in studies examining the reversibility of key events and end (adverse) effects following various periods of exposure. Weaker evidence occurs when temporal data on key events are missing.

The template presented in Table 4 is often helpful in determining the extent to which evidence fulfills consideration of dose–response and temporal concordance in WOE analysis for MOA. If the hypothesized MOA is supported, the table should fill diagonally from the top left-hand corner to the bottom right-hand corner. This "pattern" supports a continuum of the relationship between early key events occurring at lower doses than late key events and outcome. Evidence of dose–response and temporal concordance is, for example, weaker if all key events occur at all dose levels and time points. Evidence is stronger, for example, if there is a reasonable range of studies of different durations with a minimum of three dose levels each and the "pattern" of results in this table (Table 4) is as described above.

Table 4. Dose–response and temporal concordance analysis template

Temporal

Dose (mg kg⁻¹ bodyweight day⁻¹)	Key event 1	Key event 2	Key event 3

Dose-Response

Source: Meek and Klaunig (2010).

Concordance of Incidence Between Key Events and End (Adverse) Effects

- Is the occurrence of the end (adverse) effect less than that for the preceding key events?

Clear evidence of the concordance of the incidence of the end (adverse) effect with that for early hypothesized key events is influential in contributing to WOE for hypothesized MOA(s). The incidence of hypothesized early key events should be greater than that for later key events and the (adverse) outcome, consistent with the important biological underpinning that key events are essential but not necessarily sufficient, to induce the relevant end (adverse) effect. For example, the hypothesis that cytotoxicity followed by regenerative proliferation are key events in the induction of specific tumors would be supported by the observation that the incidence of the former (cytotoxicity/regenerative proliferation) is greater than that for the latter (tumors) at a similar dose. "Incidence" here refers to the occurrence of a lesion of defined severity for each of the key and end events. It should be noted that a 1: 1 correlation of the incidence of early and late key events is not anticipated; lack of evidence for a 1: 1 correlation does not detract from contribution to the overall WOE. Consistent with the essentiality (but not necessarily sufficiency) of key events, lack of 1: 1 concordance is not unexpected, being a function of biological variability; i.e., lesions will not have progressed to the end (adverse) effect in all animals at the termination of exposure.

Consistency

- Is the pattern of observations across species/strains/organs/test systems what would be expected based on the hypothesized MOA?

Evidence of internal consistency within the collective data set for a chemical contributes to increased confidence in the WOE supporting a MOA. For example, if the initial hypothesized key event is oxidative metabolism to a reactive intermediate, are the target tissues and organs those which would be expected based on knowledge of distribution of the relevant metabolic enzyme? Evidence of consistency is stronger if the pattern of species-, strain- and sex-related variations in response is what would be expected based on known differences in metabolic profiles (e.g., extent and rate of metabolism to the putatively toxic entity). Evidence is weaker if there is either significant inconsistency in the expected pattern of the collective data based on the hypothesized MOA (e.g.,. the effect or result is only demonstrated in a single rat strain when data are available for multiple strains, for all of whom metabolizing capacity for the relevant pathway is anticipated to be similar) or when there are limited data available to assess this aspect.

Analogy

- Would the MOA be anticipated based on broader chemical specific knowledge?

Convincing evidence that the hypothesized MOA is operative for a broad range of chemically similar substances also contributes significantly to WOE. For example, consider the case where reductive metabolism for chemically similar substances is associated with a particular pattern of observations leading to the end (adverse) effect. If the pattern of observations for a related chemical is distinctly different, the evidence is weaker that these effects are produced by a similar MOA. On the other hand, if there is an extensive database illustrating that the MOA of interest is operative and leads to similar end (adverse) effects for several closely structurally related chemicals as identified, for example, by (quantitative) structure–activity modeling, evidence is stronger.

The rank order of the B/H considerations suggested above reflects their relative contribution to WOE determinations of MOA and is based on evolving experience internationally. In essence, data that conflicts with a broader biological understanding ranked highly here may be grounds for considering the available supporting data as inconsistent with the hypothesized MOA, whereas lack of concordance of some empirical data is often due to variations in, for example, dose spacing or administered doses in various studies and based on careful evaluation, would not detract meaningfully from the supporting database. In assessing the totality of the WOE, it is helpful to systematically take into account all of the considerations presented here as a basis to contribute to transparency in decision making. Such assessment benefits most from multidisciplinary input from both the relevant research and risk assessment communities. However, there is no minimum number of these evolved B/H considerations that must be met to determine sufficiency and/or associated confidence but rather, in their careful, systematic, more transparent and consistent consideration, cohesiveness (or not) of the supporting data becomes evident. It is also important to recognize that while some of the evolved B/H considerations may address the association of just one key event to the end event (e.g., essentiality of key events) the WOE determination is based on consideration of the interdependence of the key and end events in the hypothesized MOA.

Comparative Weight of Evidence Case Studies

To illustrate the utility of the comparative WOE approach, assessments for two chemicals (USEPA, 2009, 2010) were selected as case studies (i.e., carbon tetrachloride and TCP). The assessment of carbon tetrachloride drew on a previous evaluation of the US EPA (Manibusan *et al.*, 2007), though the conclusions varied. These assessments were chosen based on the condition that B/H considerations for WOE had been explicitly addressed, consistent with the analysis in the MOA/HR framework for several potential MOA(s) for carcinogenicity. The focus here was not on the conclusions of the assessments; rather, the extensive review and synthesis of data therein provided the opportunity to address the potential utility of comparative analysis based on the evolved B/H considerations for WOE in MOA/HR analysis. As such, the evidence and conclusions were not re-evaluated but were simply extracted from the referenced assessments and summarized in the narrative tables presented (Tables 5a,b and 6) for the purpose of illustrating the methodology. Similarly, assessment of the underlying investigations was not considered, though based on the approach presented here, this might constitute an important next step. The literature reviews were also not updated, as the current analysis does not focus on particular chemicals but rather the potential value of the proposed methodology.

Carbon Tetrachloride

This analysis is based on a published hazard and dose–response assessment for carbon tetrachloride (USEPA, 2010). Carbon

Table 5. (a) Comparative weight of evidence analysis for carbon tetrachloride: cytotoxic MOA[a]

Evolved Bradford Hill considerations		Supporting data	Inconsistent data	Missing data
1. Biological concordance		Sustained cytotoxicity and proliferation is a well-established MOA for chemically mediated carcinogenicity.		
2. Essentiality of key events		No carbon tetrachloride induced liver toxicity in CYP2E1 knockout mice. CYP450 inhibitors prevent carbon tetrachloride liver damage. Mice treated with CYP450 inducers have increased carbon tetrachloride toxicity in subchronic and chronic studies.		
3. Concordance of empirical observations	Dose–response	Cytotoxicty and proliferation are observed at doses equal to or lower than doses at which tumors develop in rats and male mice	Tumors elevated at the lowest dose tested in female mice (5 ppm) without hepatocellular damage.	
	Temporality	Progression from cytotoxicity to hepatocellular proliferation is supported in acute and subchronic studies in rodents. Temporal relationship of cytotoxicity, repair, proliferation and tumor development is also supported in chronic cancer bioassay in rats.		Temporal relationship in female mice is not clearly defined.
	Incidence			
4. Consistency		Hepatic toxicity, necrosis and regenerative proliferation have generally been reported in animals exposed to carbon tetrachloride orally or by inhalation and are correlated with CYP450 content. Some evidence of DNA damage observed in concert with cytotoxicity.	One study reported development of tumors in mice at doses that did not produce necrosis but design of study may have influenced this result as animals were killed 1 month after last treatment.	
5. Analogy				

MOA, mode of action.
[a]All conclusions in the above tables were extracted from the original US EPA toxicology review on carbon tetrachloride (USEPA, 2010).

(b) Comparative weight of evidence analysis for carbon tetrachloride: mutagenic MOA[a]

1. Biological concordance		Genotoxic MOA is well established for chemically mediated carcinogenicity.		
2. Essentiality of key events				
3. Concordance of empirical observations	Dose–response		Genotoxicity generally found at doses with cytotoxic effects.	Measurement of genetic damage to DNA has not been well

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			characterized at dose levels that do not cause cytotoxicity.
	Temporality	Temporality not observed. Genotoxicity generally found in concert with cytotoxicity.	
4. Consistency	Incidence	Extensive *in vitro* and *in vivo* genotoxic data are primarily negative. Doses where cytotoxic events are observed are lower than doses for which mutagenicity has been evaluated. Limited positive results in genotoxicity assays appear more related to a cytotoxic response than to a mutation event	Genetically damaging events occurring at or below doses that induce cytotoxicity in laboratory rodents.
5. Analogy			

MOA, mode of action.
[a]All conclusions in the above tables were extracted from the original US EPA toxicology review on carbon tetrachloride (USEPA, 2010).

tetrachloride caused hepatocellular adenomas and carcinomas in rats, mice and hamsters in oral studies and in rats and mice following inhalation exposure. In addition to liver tumors, adrenal pheochromocytomas were observed in male and female mice following oral and inhalation exposure, for which it was concluded that data were inadequate to evaluate MOA. There was no increase in pheochromoctyomas in rats.

Based on the analysis of available data, including that on MOA, it was concluded in the assessment (USEPA, 2010) that the agent is likely a human carcinogen. Further, a potential MOA for carbon tetrachloride-induced liver tumors was hypothesized, with the following key events that included: (1) metabolism to the trichloromethyl radical by CYP2E1 and subsequent formation of the trichloromethylperoxy radical; (2) radical-induced damage leading to hepatocellular toxicity; and (3) sustained regenerative and proliferative changes in the liver in response to hepatotoxicity. The possibility that carbon tetrachloride may act via a mutagenic MOA (i.e., where mutation is an influential early key event in the induction of tumous versus, for example, being secondary to tissue damage) was also considered but not evaluated in a manner based on WOE considerations consistent with the MOA/HR framework. Based on the inconsistencies in the database supporting a potential role for the cytotoxicity, regenerative, proliferation-based MOA at the low end of the experimental exposure range and the complexity of the genotoxicity database, it was concluded that, "... the carcinogenic MOA for carbon tetrachloride is not known. Therefore, consistent with the *Guidelines for Carcinogen Risk Assessment* (USEPA, 2005b), linear low-dose extrapolation as a default approach was applied to data for liver tumors and pheochromocytomas" (USEPA, 2010).

1,2,3-Trichloropropane

This analysis is based on a hazard and dose–response assessment of TCP released in 2009 (USEPA, 2009). Based on the observed statistically significant dose-related increases in multiple tumor types in both sexes of rats and mice in a 2-year carcinogenicity assessment (NTP, 1993) and related mechanistic data (including that on genotoxicity), it was concluded that TCP is "likely to be carcinogenic to humans" via a mutagenic MOA. Relevant data for alternative MOA(s) such as cytotoxicity with tissue repair and disruption of cell signaling were considered insufficient to evaluate. It was further concluded that the available data support a hypothesized mutagenic MOA with two key events: (1) metabolism to a DNA-reactive compound, and (2) (early) induction of mutations. A low-dose linear extrapolation approach to dose–response analysis was applied, consistent with the *Guidelines for Carcinogen Risk Assessment* (USEPA, 2005b)

Comparative Weight of Evidence Analysis

Narrative comparative WOE summary tables were constructed for the hypothesized and alternative MOA(s) for carbon tetrachloride (Table 5a,b) and for a mutagenic MOA for TCP (Table 6) based on the consideration and evaluation of the data in the existing assessments (USEPA, 2009, 2010). For each postulated MOA, supporting data, inconsistent data and missing information were tabulated in the context of the evolved B/H considerations. As per MOA/HR framework recommendations, the information in the supporting and inconsistent data columns capture what has been observed, not what might be possible if more experiments had been performed. In addition, the

Table 6. Comparative weight of evidence analysis for 1,2,3-trichloropropane: mutagenic MOA

Evolved Bradford Hill considerations		Supporting data[a]	Inconsistent data[a]	Missing data[b]
1. Biological concordance		Genotoxic MOA is well established for chemically mediated carcinogenicity		
2. Essentiality of key events		Inducers/inhibitors of metabolism alter amount of DNA binding		Evidence for adduct conversion to genetic damage
3. Concordance of empirical observation	Dose–response	Dose-related formation of DNA-reactive metabolite, DNA adduct formation, tumor formation and time to tumor.		
	Temporality	Metabolism to reactive intermediate occurs within hours of exposure, adducts appear within hours and days of exposure, and tumors first appear after ≈ 9 months.		
	Incidence			No data to assess whether adduct formation frequency different from tumor frequency.
4. Consistency		Mutagenic effects *in vitro* accompanied by limited evidence of *in vivo* mutagenicity.	Adducts occur in tissues where no neoplastic effects were reported (spleen, liver and glandular stomach). Negative results from *in vivo* genotoxicity assessments (dominant lethal and micronucleus).	
5. Analogy		Other halogenated aliphatic chemicals (1,2,-dibromoethane and 1,2-dibromo-3-chloropropane) are mutagenic carcinogens. Other genotoxic chemicals are multisite and multispecies carcinogens.		

MOA, mode of action.

[a]All conclusions in the above tables were extracted from the original US EPA toxicology review on 1,2,3-trichloropropane (USEPA, 2009).

[b]The IRIS assessment did not comment on missing data; the information here represents the authors' views.

information noted in the missing column only includes that which is testable and important for informing the MOA (i.e., critical data needs). Ideally, a discussion on whether the missing information is critical and would detract from or impact conclusions regarding the proposed MOA should accompany this comparative WOE table. Blank cells would typically represent instances where data either do not exist or are inadequate for evaluation. However, in this case, as the analysis draws upon an existing assessment, blank cells may also represent where text was either absent or inadequate to address the evolved B/H considerations.

Qualitative Assessment of Overall Evidence

For both case studies, the focus is not to conclude on the sufficiency of underlying data to support a particular MOA conclusion, but rather to illustrate the utility of the comparative WOE approach for increasing transparency in the assimilation of data.

Visually, Tables 5(a,b) and 6 highlight the availability of supporting and discrepant data on the MOA(s) evaluated for carbon tetrachloride and TCP. Comparative WOE analysis, for the two hypothesized MOA(s) for carbon tetrachloride based on the published assessment (USEPA, 2010), indicates that the supporting data for the hypothesized MOA involving cytotoxicity (necessarily within the range of experimental observation) fulfill a number of the evolved B/H considerations. This contrasts with the comparatively more limited support for the hypothesized mutagenic MOA. This difference highlights:

(1) the potential utility of comparative analysis for assessing the WOE of alternative MOA(s) for individual chemicals, based on the evolved B/H considerations to more explicitly indicate the degree of confidence in a particular MOA, and
(2) the desirability, in the interest of transparency and consistency, of separating conclusions reflecting assessment of the relative WOE for MOA in the observable experimental range based on articulated and explicit considerations from those based on inference or extrapolation to the low-dose range. It is anticipated that such an approach has the potential to increase transparency in delineating science judgment determinations from those related to public policy.

The comparative WOE analysis for TCP also provides a basis for comparison across chemicals of a relatively strong database for a mutagenic MOA, which can be contrasted with one that is relatively weak, potentially as a basis to increase consistency in determinations. In this case, perspective on the degree of confidence in the supporting WOE for the hypothesized mutagenic MOA for carbon tetrachloride (Table 5b) can be gained through comparison with the nature and extent of data available for the stronger database for TCP (Table 6).

Discussion

Comparative aspects of WOE analyses are illustrated here as a basis to contribute to transparency and consistency in delineating confidence/uncertainty in MOA/HR analysis based on the BH considerations. As noted by Guyton *et al.* (2008), Hill's (1965) considerations were not developed originally for evaluation of experimental/mechanistic data, though their utility for application in modified form to assess WOE in MOA analysis has been repeatedly though inconsistently tested. Based on increasing experience internationally in MOA/HR analysis (see, for example, Boobis *et al.*, 2006, 2008, Meek *et al.*, 2014), evolved B/H considerations are proposed here and clarified through delineation of the specific aspects addressed by each as framed by a series of questions. Definitions for these considerations have been additionally simplified and tailored to application in MOA analysis. The evolved B/H considerations were also rank ordered to reflect their relative contribution to WOE determinations and their utility exemplified in a comparative WOE approach.

The evolved B/H considerations build on previously published iterations and reflect experience in the application of MOA analysis. Several terms were clarified to facilitate assimilation of relevant chemical specific and biological data (i.e., "specificity" is now termed "essentiality of key events," "biological plausibility and coherence" is now termed "biological concordance" and concordance of empirical observations among key events delineated). In addition, considerations with limited relevance for evaluating MOA data (i.e., "strength," "coherence" and "experiment") were eliminated while other considerations (i.e., "analogy" and "incidence concordance") were added based on evolving experience with larger numbers of chemicals. It is hoped this evolved terminology, which reflects more common understanding within the broader risk assessment (versus epidemiological) community, will additionally contribute to consistency of use in MOA analysis. Finally, considerations were redefined as a basis to promote consistency and utility. For example, in publications of the IPCS MOA/HR framework (Boobis *et al.*, 2006, 2008; Sonich-Mullin *et al.*, 2001), consistency is defined as repeatability of key events in different studies; while in the USEPA cancer guidelines, consistency refers to the pattern of elevated risk observed across several independent studies (USEPA, 2005b). Neither definition accurately reflects the use of consistency in evaluating the WOE for hypothesized MOA(s). The former simply assesses reproducibility of results and, as such, may only contribute to the level of confidence in the occurrence of one key event. The latter definition is more appropriate to the assessment of the reproducibility of results in epidemiological and not mechanistic data sets. Consistency in the context of the MOA/HR framework more appropriately relates to evaluation of the WOE supporting interdependence of the key and end (adverse) events. Therefore, consistency was redefined here to reflect support of the pattern of effects across species/strains/organs and test systems for the hypothesized MOA. For example, if metabolism is a hypothesized key event in a carcinogenic MOA, the pattern of species-, strain- and sex-related variations in tumor response is compared to that expected based on known differences in metabolic profiles in the test systems. As such, it is not as important to assess if the occurrence of tumors is reproducible across studies, but rather, if the presence or absence of tumors in various species and strains is consistent with the hypothesized MOA.

Comparative WOE analysis is illustrated as a means of increasing understanding of the nature of transparency that is essential when evaluating confidence in the supporting WOE for hypothesized (potentially competing) MOAs. In doing so, it also provides a basis for increasing consistency in evaluation. Presentation of an overview of the data in a comparative manner (i.e., as supporting, inconsistent and missing) based on templates that cue evaluators concerning critical aspects provides concise insight into the extent of available data and relevant patterns in the existing database, which support various levels of confidence in considered options. In addition, this presentation concisely communicates areas of uncertainty (inconsistent data column and blank cells) and highlights areas of greatest impact for future research (missing data column). Ideally, further transparency on the impact of this information (i.e. supporting, inconsistent and missing data) on the MOA conclusions would be provided in a detailed, supplemental discussion.

Synthesis of a collective data set to evaluate WOE for a hypothesized MOA is complex and challenging, requiring multidisciplinary input from both the research and risk assessment communities. This analysis is dependent upon transparent and consistent evaluation of the extent and nature of both chemical-specific and biological data versus supposition about possibilities for which there is essentially no experimental support. Characterization of the evolved B/H considerations is anticipated to contribute to more robust and transparent analyses, as a basis also to discourage, without clear rationale, the discounting of well-supported options based on the emphasis of outlying data of lesser quality.

This manuscript extends MOA/HR assessment through evolution of the B/H considerations and illustration of a comparative WOE analysis. Ultimately, it is anticipated that the additionally articulated and comparative aspects, which build on considerable recent experience in MOA analysis, will contribute to increasing transparency, consistency and methodological rigor in separating aspects of science judgment (i.e., weighting of options based on transparent consideration of available scientific support) from those of public policy in regulatory risk assessment (the latter of which sometimes involves embedded conservatism, to increase public health protection).

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This work builds on previous products of a significant number of contributing authors and collaborators who have participated in the development and application of mode of action/species concordance analysis and associated training materials. Contributors include those outlined in Meek *et al.* (2003, 2014), Seed *et al.* (2005) and Boobis *et al.* (2006, 2008).

Conflict of Interest

Several of the authors (C.M.P., A.N.B., C.M.N. and R.J.L) are employed by a subsidiary of Exxon Mobil, who produces materials evaluated by the US EPA. Methodological aspects based on case studies considered here do not relate to specific evaluations of relevance to Exxon Mobil.

The work reported in the paper was conducted during the normal course of research/training for the University of Ottawa (MEM). It evolved from discussions during and following provision of training, for which funding for development of materials, travel and lodging was provided.

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Bohn, Brent 1216

From: Lee, Janice
Sent: Monday, September 15, 2014 11:14 AM
To: Powers, Christina; Cowden, John
Subject: sot abstract
Attachments: SOT 2015 Lee et al_9 12 14.docx

Hi Christy and John,

Attached is a very rough draft for SOT. I welcome all suggestions and feedback.
It's always a challenge to try and say something, when that something hasn't been done yet. ☺

Thanks, Janice

Bohn, Brent 1225

From: Cowden, John
Sent: Tuesday, September 09, 2014 4:09 PM
To: Powers, Christina
Cc: Lee, Janice; Joca, Lauren
Subject: Revised lit search figure
Attachments: Gen lit search diagram - draft.pptx

Hi Christy,

Happy Tuesday! I hope that things are going well for you today up in Ann Arbor. Take solace that no matter how bad it is to lose to ND, things are worse in Columbus!

I've put together a simplified lit search diagram for the ADP revisions, based on your version. I wanted to get your input, particularly on the MOA stuff. The goal of this figure is to be generic enough to cover both hazard ID and DR.

Let me know if you have any suggestions. Have a great afternoon!

John

John Cowden, Ph.D.
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National Center for Environmental Assessment (NCEA)
U.S. Environmental Protection Agency - RTP
(919) 541-3667

Bohn, Brent 1227

From:	Powers, Christina
Sent:	Monday, September 08, 2014 4:02 PM
To:	Philpott, Olivia
Cc:	Powers, Christina
Subject:	Room B249 Reservation request
Follow Up Flag:	Follow up
Due By:	Wednesday, September 10, 2014 7:30 AM
Flag Status:	Flagged

Hi Olivia,

Is B249 available on Monday, Sept. 15th from 9:30-10:30? If so, can you add my name to the room reservation for that time period? The arsenic adverse outcome pathway team needs a room with projector capability.

As always, don't hesitate to contact me with any questions or concerns.

Thanks!
Christy